Project report: International Collaborative Trial of a Real-Time PCR Method for the Relative Quantitation of Horse DNA

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Provision of LGC Services for the organisation and running of an International Collaborative Trial

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Contact: Malcolm Burns
Tel: 0208 943 7000

Prepared by: Timothy Wilkes and Malcolm Burns

Approved by: Peter Bedson

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1. Executive summary

Following the UK/EU Horse-meat issue of 2013, where a significant amount of horse DNA was found in a beef burger product on sale at a supermarket store, a real-time PCR approach for quantitation of horse DNA was developed at LGC using Defra funding. This method subsequently underwent an in-house validation in order to provide evidence of its fitness for purpose using meat derived from different horse samples, to characterise the precision around the 1% (w/w) level for enforcement action, and to assess its applicability to processed food materials.

An international collaborative trial of the method (based on IUPAC and ENGL guidelines) [1,2] was organised in order to evaluate the repeatability and reproducibility of the method within and between laboratories. A total of seventeen laboratories participated in the trial, representing nine official control laboratories and private labs in the UK, six laboratories from EU member states, and laboratories in Switzerland and the United States of America.

All participants were provided with a copy of detailed working instructions, a pro-forma for recording and returning results, as well as all of the necessary samples and reagents for the analyses (inclusive of test samples and positive/negative controls). Each of the participating laboratories were requested to return results based on the evaluation of five blindly labelled test samples (representing 0.1, 0.5, 1, 5, and 20 % (w/w) horse meat in a beef meat background) relative to a calibration curve. Test samples were provided as DNA extracted from gravimetrically prepared raw horse-meat in a raw beef background. Each sample was represented by four units in the experimental design, and each unit represented by triplicate PCR technical replicates.

The collated data from the collaborative trial was subject to statistical analysis, and significant outliers removed from subsequent analysis. Based on the remaining data, the mean values for the PCR efficiency and r-squared associated with the calibration curves for the horse assay were 94.1% and 0.998 respectively, and for the mammalian assay, 96.4% and 0.997 respectively. The values obtained provided evidence that the PCR efficiency and linearity for the calibration curves across all laboratories was good.

A mixed effects model, based on maximum likelihood, was used to estimate the variance associated with the laboratories, replicate plates, replicate samples within the same sample level, and the residual variance. The relative repeatability standard deviation (RSD_r) was calculated as less than 9% across all test samples for the levels 0.5% to 20% (w/w) inclusive, and as 15% at the 0.1% (w/w) level. The relative reproducibility standard deviation (RSD_R) was calculated as less than 18% across all test samples for the levels 0.5% to 20% (w/w) inclusive, and as 26% at the 0.1% (w/w) level. These values fulfil the acceptance criteria for the precision associated with a method subject to a collaborative trial as outlined in published ENGL guidance notes for minimum performance requirements for analytical methods [2]. Recent

DNA sequencing projects have provided size estimates for the equine and bovine genomes, which can be used to calculate the assigned values (on a copy number basis) of the nominal w/w samples. A small but consistent positive bias between 8 and 15% compared to the assigned value was observed across all sample levels evaluated in the collaborative trial, thought to be mainly contributed to by the sample preparation approach.

Both the repeatability and reproducibility estimates from the collaborative trial provide evidence for the good precision of the method within and between laboratories. Given the good precision and trueness estimates associated with this method as evidenced by an international collaborative trial, it is a recommendation of this project that the method be considered for standardisation at an international level.

Routine sample analysis using this method could benefit from an improved experimental efficiency (e.g. more efficient plate design, development of multiplex assays) which would enable higher throughput with an associated decrease in sample costs. Furthermore, recent advances in DNA sequencing may facilitate better assignment of gravimetric w/w test samples in terms of mammalian copy numbers. Incorporation of such knowledge in future tests may facilitate even more accurate estimation of the meat content of samples (for example, using digital PCR). It is a recommendation from this project that such value assignment be considered for the future.

2. Introduction

Food authenticity and food fraud are becoming increasingly prevalent within the European food industry, partly due to the pressures faced by producers within today's challenging financial climate and also the international nature of modern food production. The recent EU wide issue involving the detection of the undeclared presence of horse-meat in beef products destined for human consumption [3] has emphasised the need for the development of accurate analytical approaches for the quantitative detection of meat adulteration in a sample.

In response to the EU horse-meat issue, a real-time PCR method was developed at LGC for the quantitation of horse DNA (Defra project FA0135) [4]. The method was demonstrated to be capable of detecting and accurately quantitating the amount of horse DNA present in samples of raw, lean beef muscle mixtures. The method has been validated through an objective assessment of performance characteristics using DNA:DNA ad-mixtures, and weight for weight (w/w) gravimetric, raw horse-meat in raw beef (meat) materials. Additional method validation performed in a subsequent Defra project (Defra project FA0146) [5] showed the applicability of the method using different horse samples, evaluated the measurement uncertainty around the 1% (w/w) threshold level for enforcement action, and provided evidence of the method's suitability for use in complex foods [5]. These two Defra projects [4,5] resulted in the generation of a standard operating procedure (SOP) for the

method (Draft Standard Operating Procedure For The Quantitation Of Horse DNA Relative To Mammalian DNA In Raw Meat Samples) [6], and a peer reviewed paper describing the approach (Nixon *et al*., 2015) [7].

In order to independently assess the performance of the method between laboratories, an international collaborative trial of the method was conducted. The trial was designed and implemented in accordance with guidance available from the International Union of Pure and Applied Chemistry (IUPAC) (IUPAC protocol for the design, conduct and interpretation of method-performance) [1] and European Network of GMO laboratories (ENGL) (Definition of minimum performance requirements for analytical methods of GMO testing) [2].

The resulting international collaborative trial for the evaluation of the real-time PCR method involved the co-operation of seventeen laboratories from the United Kingdom (UK), additional European Union (EU) member states, Switzerland (CH), and United States of America (USA). This report summarises the implementation of the trial, the results obtained, and the analysis of the performance of the method.

3. Methods

3.1. Selection of participating laboratories

As part of this international collaborative trial, the method was tested in seventeen laboratories in order to determine its performance. Invitations to participate in the trial were sent to all of the UK Official Control Laboratories, laboratories representing the European Network of GMO Laboratories (ENGL), as well as several commercial testing laboratories located in the UK, EU and USA. In total, forty-six laboratories expressed their willingness to participate in the trial, and provided LGC with details relating to their previous experience with real-time PCR. Selection of laboratories to participate was based on: (i) an evaluation of their previous experience in the field of food authentication testing using real-time PCR; (ii) real-time PCR platform(s) available; and (iii) participant certification/accreditation to recognised quality measurement systems (e.g., ISO 9001:2008 [8], ISO 17025:2005 [9]). Seventeen laboratories participated in the trial, comprising six UK Public Analyst laboratories, two UK private companies, six laboratories from other EU member states, one laboratory from Switzerland, one laboratory from the USA and LGC as the lead laboratory. Guidance was provided to all of the selected laboratories with regards to the Standard Operating Procedure to be followed, as well as all of the samples and reagents required for the execution of the method. A list of laboratories which participated in the collaborative trial is provided in **Table 1**.

3.2. Sample preparation

Raw muscle tissue (horse and beef meat) which had been trimmed free of surface inter-muscular fat and connective tissue was sourced from a reputable supplier (Kezie Ltd, Duns, UK) and authenticated as to species type.

3.2.1. Authenticity testing

Species identity of meat samples was authenticated in-house using real-time PCR and DNA sequencing-based approaches:

- Real-time PCR: Authenticity of the supplied raw meat was validated based on the amplification of species specific genomic targets using PCR assays specific for horse (Köppel *et al*.,2011) [10] and beef (Laube *et al*.,2003) [11].
- DNA analysis: Authenticity of the supplied raw meat samples was additionally validated through the DNA sequence analysis of specific gene targets. Two mitochondrial genes (12S rRNA and cytochrome b) were used for the species specific identification of each joint of meat. Universal PCR primers which anneal to conserved regions of the 12S, and cytochrome b mitochondrial genes were used to synthesise PCR products, for which the DNA sequence was subsequently derived by Sanger sequencing. Species authenticity was then confirmed with use of the Basic Local Alignment Search Tool (BLAST) [12] available at the website of the National Centre for Bioinformatics (NCBI) [13], and species identity confirmed using sequence information available on GenBank [14].

Table 1: Laboratories which participated in the validation of a real-time PCR method for the quantitation of horse DNA.

Laboratory	Country
European Union reference laboratory for animal proteins in feeding stuffs (EURL-AP)	Belgium
Bavarian Health and Food Safety Authority	Germany
European Union Reference Laboratory for Genetically Modified Food and Feed, European Commission, Directorate Joint Research Centre (JRC), Directorate F - Health, Consumers & Reference Materials	Italy
State Veterinary and Food Institute Dolný Kubín	Slovakia
GMO group, National Institute of Biology, Department of Biotechnology and Systems Biology	Slovenia
National Centre for Food, Spanish Food Safety Agency and Nutrition (CAN-AESAN)	Spain
Federal Department of Economic Affairs, Education and Research EAER ,Institute for Lifestock Sciences	Switzerland
Dundee City Council Scientific Services	United Kingdom
Edinburgh Scientific Services	United Kingdom
Fera Science Ltd.	United Kingdom
Glasgow Scientific Services	United Kingdom
Lancashire County Scientific Services	United Kingdom
LGC Ltd.	United Kingdom
Minton, Treharne and Davies Ltd.	United Kingdom
Premier Analytical Services	United Kingdom
Worcestershire Scientific Services	United Kingdom
Q Laboratories, Inc.	United States of America

3.2.2. Test sample generation

Test samples were produced for mass-based ratio preparations of: 100%, 20%, 5%, 1%, 0.5% and 0.1% weight for weight (w/w) raw horse muscle tissue in a raw beef muscle tissue background. All five sample levels were produced according to the following procedure. Raw muscle tissue (horse meat) which had been trimmed free of surface inter-muscular fat and connective tissue was cubed, thoroughly homogenised in a new species specific food processor, combined and then mixed and the resultant paste stored in polythene bags on ice until required. The same approach was taken for the preparation of the beef (meat), as had been used for the horse material. For the 20% and 5% w/w samples, an appropriate mass of the 100% homogenised raw horse meat was weighed using a UKAS certified calibrated top-pan balance (accurate to two decimal places) and combined with an appropriate mass of the 100% w/w beef (meat). To ensure the accuracy of mass based ratio mixes the top-pan balance reading was allowed to stabilise to room temperature, and where necessary additional material was added to or removed from with the aid of a micro-spatula to produce the weights accurate to two decimal places shown in **Table 2**. In order to provide representative low levels, for the 1.0%, 0.5% and 0.1% w/w samples, an appropriate mass of the homogenised 5% w/w raw horse was combined with an appropriate mass of the 100% w/w beef (meat). Details of the sample composition for the five levels of horse DNA used are listed in **Table 2**. Each of the samples was thoroughly mixed in a clean food processor, three aliquots of 1 g set aside for use with DNA extraction, and the remaining materials each double bagged in zip lock polythene bags and stored at -80^{o}C.

Table 2: Generation of gravimetrically prepared admixes (top pan balance accurate to two decimal places).

Test Sample	100% Beef (g)	100% Horse (g)	5% Horse in Beef (g)	Total Mass (g)
100% w/w Beef	2000	0	0	2000
100% w/w Horse	0	2000	0	2000
20% w/w Horse in Beef	160	40	0	200
5.0% w/w Horse in Beef	190	10	0	200
1.0% w/w Horse in Beef	160	0	40	200
0.5% w/w Horse in Beef	180	0	20	200
0.1% w/w Horse in Beef	196	0	4	200

3.3. DNA extraction

For the comparative trial, DNA extraction was performed on 1 g tissue samples using the modified CTAB extraction method of Binke *et al*.(2003) [15]. Purified DNA was suspended in 0.5 ml of nuclease free water and both DNA yield (A_{260}) and quality characteristics ($A_{260:230}$ and A_{260}:A_{280}) determined with use of a Nanodrop™ ND-1000 Spectrophotometer (Thermo Scientific, UK). Suitability of the extracted DNA for use with real-time PCR was performed using the horse specific (*EC-GHR1*) and universal mammalian (MY) real-time PCR assays. Performance was evaluated

through examination of the resulting PCR efficiencies, and the linearity of the data obtained (R^2).

3.4. DNA Calibration standards

DNA extracted from 100% w/w raw horse meat was used as the calibrant for the generation of standard curves for the universal mammalian and equine specific real-time PCR assays as specified in the Standard Operating Procedure (SOP) (**Annex 3**). The initial DNA concentration was estimated with use of spectrophotometry (OD_{260}). Dilution to a suitable working concentration was performed with the addition of an appropriate volume of EB buffer (Qiagen). Participants were provided with detailed instructions on the preparation of a five point, seven fold dilution series which encompassed a genome equivalent copy number range from 24,010 copies/µL, to 10 copies/µL. A copy of the working instructions circulated to participating laboratories has been included in **Annex 4**.

3.5. Test unit preparation

The initial DNA concentration of the five gravimetrically prepared samples were determined spectrophotometrically (OD_{260}) as previously described.

Twenty blinded samples (labelled from U1 to U20), representing five different levels of horse DNA were used in the collaborative trial. The levels of horse DNA used were extracted from gravimetrically prepared samples of 0.1%, 0.5%, 1.0%, 5.0% and 20.0% of raw horse meat in a raw beef background. Each level of horse DNA was replicated four times: two of the technical replicates were randomly assigned to test samples between U1 to U10, and the remaining two technical replicates were randomly assigned to test samples between U11 and U20. All test samples were stored at – 80^{o}C until they were shipped on dry ice by courier to participants.

3.6. Test unit stability and homogeneity evaluation.

Prior to shipment, identical samples were tested at three different time points (T1, T2, T3) and in accordance with the working instructions that had been provided to all participating laboratories. The time points for the evaluation represented 1, 14 and 62 days after generation and freezing of the test units (corresponding to critical times for dispatching samples for the pre-trial and main trial). The resulting quantitative data was jointly used to infer the short term stability of the reagents and materials provided, as well as the homogeneity of each of the test units U1 to U20.

3.7. Real-time PCR

Real-time PCR was performed as described in the SOP (**Annex 3**), and as specified in the working instruction provided to all participating laboratories (**Annex 4**). Essentially, two Taqman real-time PCR assays were run, one targeting a region within the equine growth hormone receptor gene (*EGHR1*) (Köppel *et al*., 2011) [10], and the second targeting a region within the mammalian myostatin gene (*MY*) (Laube *et al*., 2003) [11]. For the relative quantitation of the horse DNA in an unknown sample, a seven-fold, five point serial dilution of the calibrant (supplied) was performed, which was then used as the template for the two real-time PCR assays in order to generate two standard curves for a fixed mass of input DNA. Each

of these calibration curves represents the approximate genomic equivalent copy number of either the total mammalian or total horse DNA present. The mass, and hence the relative copy number of both horse, and mammalian DNA can therefore be determined for the unknown sample by comparing the C_q obtained for each assay with the calibration curves that had been generated with use of the calibrant. The contents of the calibration samples are provided in **Table 3**.

3.8. Experimental design

Participants were required to set-up and analyse two 96 well PCR plates, or the equivalent. For each PCR plate, the samples were analysed using both the equine growth hormone receptor 1 specific assay (*EC-GHR1*), and the myostatin (*MY*) universal mammalian assay.

3.9. Collaborative trial

For the international collaborative trial seventeen laboratories participated (**Table 1)**. The collaborative trial was designed in accordance with IUPAC and ENGL guidelines for the design, conduct and interpretation of collaborative studies [1,2]. Participating laboratories were provided with 20 coded (blind) DNA samples to analyse using the real-time PCR method, representing the five levels of raw horse meat in a raw beef meat background. The use of five levels, as recommended in the IUPAC guide [1], enabled an assessment of the usefulness of the test method at various levels of adulteration, and provided data for the estimation of levels of repeatability precision (S_r; RSD_r) and reproducibility precision (S_R; RSD_R). To minimise inter-laboratory variability (not attributable to the method), all of the required reagents were provided, with the exception of nuclease free water.

3.10. Protocol for the collaborative trial

All participants received electronic copies of detailed working instructions for performing the method, plus an electronic copy of an Excel (Microsoft®) pro/forma reporting sheet to be returned to LGC for statistical analysis. Participants were also required to report, in detail, any additional information that could have influenced their results, including: (i) make and model of real-time PCR instrument used, (ii) date of instrument calibration, (iii) level of accreditation held by laboratory (e.g., ISO 17025), and (iv) the number of analysts involved in the trial. In addition, participants were required to return a file containing the raw data generated from each of the PCR experiments performed. A copy of the working instructions circulated to all of the participants is shown in **Annex 4**.

Table 3: Genome equivalents and mass of DNA present in each of the experimental test samples.

	Sample				
	S1	**S2**	**S3**	**S4**	**S5**
Genome equivalents (copies)	24,010	3,430	490	70	10
Mass of horse DNA in each reaction (g)	1.30 E-07	1.86 E-08	2.66 E-09	3.80 E-10	5.42 E-11

PCR was performed in triplicate for each of the test samples (U1 to U20) provided. Test samples were assigned to two, ninety-six well PCR plates, such that each level of horse DNA was replicated twice on each plate and in total four replicates for each level of horse DNA were analysed. Details of the plate layouts used are illustrated in **Figures 1.1** and **1.2**.

Figure 1.1: Plate set-up and loading order for Plate A (Samples U1 – U10).

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
B	STD5	STD5	STD5	NTC	NTC	NTC	U1	U1	U1	U2	U2	U2
C	U3	U3	U3	U4	U4	U4	U5	U5	U5	U6	U6	U6
D	U7	U7	U7	U8	U8	U8	U9	U9	U9	U10	U10	U10
E	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
F	STD5	STD5	STD5	NTC	NTC	NTC	U1	U1	U1	U2	U2	U2
G	U3	U3	U3	U4	U4	U4	U5	U5	U5	U6	U6	U6
H	U7	U7	U7	U8	U8	U8	U9	U9	U9	U10	U10	U10

Upper half: horse specific (EC-GHR1) assay

Lower half: universal mammalian (MY) assay

Figure 1.2: Plate set-up and loading order for Plate B (Samples U11 – U20).

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
B	STD5	STD5	STD5	NTC	NTC	NTC	U11	U11	U11	U12	U12	U12
C	U13	U13	U13	U14	U14	U14	U15	U15	U15	U16	U16	U16
D	U17	U17	U17	U18	U18	U18	U19	U19	U19	U20	U20	U20
E	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
F	STD5	STD5	STD5	NTC	NTC	NTC	U11	U11	U11	U12	U12	U12
G	U13	U13	U13	U14	U14	U14	U15	U15	U15	U16	U16	U16
H	U17	U17	U17	U18	U18	U18	U19	U19	U19	U10	U10	U10

Upper half: horse specific (EC-GHR1) assay

Lower half: universal mammalian (MY) assay

3.11. Data analysis

Both the raw data and the real-time PCR analyses submitted from participating laboratories were evaluated. Data for inclusion in further statistical analysis was filtered according to the following criteria: (a) laboratories reporting that they deviated from the Standard Operating Procedure provided; (b) presence of any PCR products in the negative controls; (c) PCR efficiencies outside of 100 ± 15% for either assay. The remaining data was then converted into tab-delimited text format in preparation for statistical analysis using the R statistical computing package (version 3.01).

Box plots were generated for the data and a preliminary inspection of the data performed in order to determine the presence of outlying data points. The statistical status of putative outlying data points was confirmed with use of a Grubb's test [16], and any significant outlying values removed.

Values for the repeatability and reproducibility of the method were calculated for each level with use of a mixed effects model, based on maximum likelihood. The

model used specified three random effects which were: (a) unit-to-unit variation (where each sample level was represented by four identical test units randomly assigned from U1 to U20); (b) between-plate effect nested within laboratory; (c) between-laboratory variation. Output from the model was used to derive precision estimates of the relative repeatability standard deviation RSD_r (%) and the relative reproducibility standard deviation RSD_R (%). In addition to estimates of precision for the method, performance characteristics for both real-time PCR assays were also determined. These included: (a) estimation of PCR efficiencies; (b) calculation of the R^2 values (describing the linearity of the data).

4. Materials provided

DNA samples were provided to the participating laboratories as the samples for analysis. The nature of the samples provided (DNA) was chosen on the basis of minimising problems with interference from sample extraction, mitigating against stability issues of meat samples, and administrative problems with sending meat as a sample for an international collaborative trial. The real-time PCR method measures the relative DNA content of a sample, and the purpose of the international collaborative trial was to evaluate the method itself and not the performance of the laboratories in terms of their ability to extract DNA. A number of international validation trials which are currently in effect also use this premise where DNA is provided to participating laboratories as the template [17].

For the purposes of the international collaborative trial, participants were provided with the following:

4.1. Test samples and calibrant

- Test samples provided comprised of twenty blinded genomic DNA samples, labelled U1 to U20. These had been extracted from gravimetric preparations of different percentages (0.1%, 0.5%, 1%, 5% and 20%) of lean raw horse muscle meat, in a lean raw beef muscle meat background.
- The calibrant provided comprised of two tubes of a DNA solution for the preparation of the standard curves, and consisted of genomic DNA extracted from lean raw horse muscle meat.

4.2. Reagents

Participants also received the following materials:

4.2.1. Reaction reagents

- 2x TaqMan® Universal Master Mix (Life technologies, Part#4304437): (Universal PCR Master Mix)

4.2.2. Primers and probes (1 tube each)

- *MY* (Universal mammalian) assay
  [Note: the *MY* probe is FAM labelled and BHQ1 quenched]
- *EQ-GHR1* (Equine specific) assay
  [Note the *EQ-GHR1* probe is FAM labelled and BHQ1 quenched]

5. Results

5.1. Sample authenticity

DNA sequence analysis for the mitochondrial 12S and cytochrome b gene targets confirmed the authenticity of the meat samples sourced from Kezie Ltd (UK), the result of which were further supported by data from the application of the horse specific (*EC-GHR1*) and universal mammalian (*MY*) real-time PCR assays (data not shown).

5.2. DNA extraction

DNA was extracted and purified from all of the samples for each level of horse meat (w/w) used. Good yields were observed for all of the samples and values for the A_{260}:A_{280} and A_{260}:A_{230} measurements which are indicative of sample purity were within the required criteria. Information regarding the criteria for extracted DNA quantity has been summarised in **Annex 2** of this document. DNA yield was at least 240 ng/ul in a final elution volume of 500 µl**.**

5.3. Assessment of test units stability and homogeneity

A statistical analysis was performed for the quantitative real-time PCR data generated for the pre-shipment quality control of material and reagents, across three consecutive time points (corresponding to 1, 14 and 62 days following test unit generation and freezing for storage). The pre-shipment testing was undertaken in order to determine the existence of any significant difference in the mean values obtained for each of the five sample levels (20%, 5%, 1%, 0.5% and 0.1% horse DNA) between sampling time points. At each time point, four replicates of each sample level were analysed (**Annex 5**). A one-way ANOVA was conducted for each of the five sample levels and the results obtained indicated that no significant differences were observed between time points (data not shown).

5.4. Real-time PCR assay performance

Based on participants data, the mean PCR efficiencies of 94.1% and 96.4% were determined for the horse specific (*EC-GHR1*) and universal mammalian (*MY*) assay data sets respectively, corresponding to slopes of the line of -3.476 and -3.418 respectively. Mean values for R^2 were determined as being 0.998 and 0.997 for the horse specific (*EC-GHR1*) and universal mammalian (MY) assays respectively. The corresponding PCR performance data is available in tabular format in **Annex 7**.

5.5. Statistical analysis of quantitative data

5.5.1. Preliminary inspection of data

Seventeen laboratories participated in the collaborative trial (**Table 1**). Initial quality filtering of the data was performed on a laboratory by laboratory basis. Data sets received from four of the responding laboratories failed to either meet accepted PCR performance criteria (**Annex 6**) inclusive of exhibiting poor PCR efficiency, or the laboratories encountered technical issues with the instrument, and were thus removed from further analysis.

The retained filtered data sets from the remaining 13 laboratories were combined, and used in all of the subsequent statistical analysis. Box plots for all five levels of adulteration for the combined data set are shown in **Figure 3**. Initial visual inspection of these plots suggest that: (a) plate and lab variation appear consistent across all levels; (b) test unit-to-test unit variation appears small compared with plate-to-plate and lab-to-lab variation; (c) a general positive bias exists with respect to the nominal level. Notable outlying data points were detected for laboratory 10 at the 5% level for horse DNA, and for laboratory 17 at the 1% level for horse DNA **(Table 4). (**For traceability purposes, laboratories 10 and 17 correspond to laboratories F and K respectively in **Annex 8**.)

Examination of the underlying C_q triplicate data revealed that the results for the test unit U7 (5% w/w) discrepancy observed with laboratory 10 were dispersed to an unacceptable extent, making this data point unreliable. In addition, applying Grubbs' test to the four lab 10 data points was strongly significant (p = 0.0072). This, together with the poor triplicate variability, was considered sufficient reasons to exclude the test unit U7 value from the laboratory 10 data set.

Table 4 Data points initially identified from the plot as requiring further investigation.

Laboratory	Nominal level (% horse DNA)	Plate	Unit	Measured level (% horse DNA)	Grubbs' test (*p*-value)
10	5%	1	U7	16.92	0.0072
17	1%	1	U9	1.72	0.053

The test unit U9 data point discrepancy associated with the Laboratory 17 data set was not identified as a statistical outlier and was retained (p = 0.053, Grubbs' test).

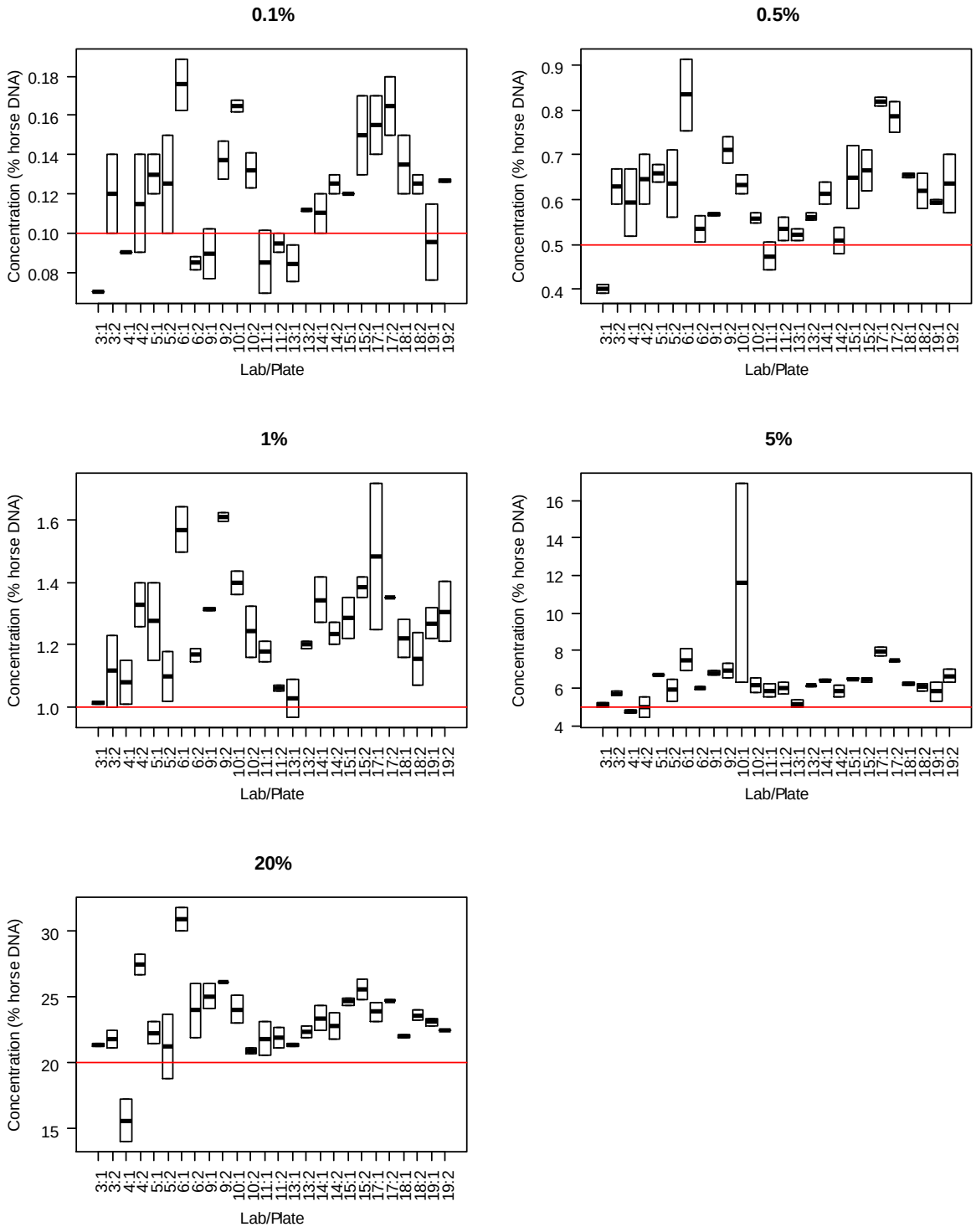
5.5.2. Quantification of horse DNA

Results returned by those laboratories which participated in the trial are summarised in **Table 5**, which was derived from the raw data (**Annex 8**). The values represent the quantitative estimates associated with the five levels of test samples (20%, 5%, 1%, 0.5% and 0.1% (w/w) horse meat).

Table 5: Summary of the quantitative results returned by participating laboratories. The values represent the results derived for the filtered data set, with technical deviants, poor PCR performance, and statistical outliers removed. Values for laboratory, plate, test unit and residual standard deviation were derived with use of the maximum likelihood model used for statistical analysis.

	Sample nominal value (% horse meat w/w)				
Metric	**0.10**	**0.50**	**1.00**	**5.00**	**20.00**
Mean estimated quantitative value of sample	0.12	0.62	1.26	6.20	23.26
Laboratory standard deviation	0.01	0.05	0.08	0.62	0.00
Plate standard deviation	0.02	0.08	0.11	0.36	2.54
Test unit standard deviation	0.00	0.02	0.00	0.00	0.00
Residual standard deviation	0.02	0.05	0.11	0.41	1.34

Figure 3. Measured concentration (% horse DNA) shown by lab and plate. Two units were each measured once on each plate. Nominal concentration is indicated by the red line.



5.5.3. Statistical analysis of measurement data

The repeatability and reproducibility were calculated for each sample level with use of a mixed effects model, based on maximum likelihood. The model specified three random effects which were: (a) unit-to-unit variation; (b) between-plate effect nested within laboratory; (c) between-laboratory variation. For the purposes of the current report, repeatability was defined as the standard deviation between repeat measurements taken by the same analyst in the same laboratory using the same instrument and corresponds to the residual standard deviation in the specified model. Reproducibility was defined as the standard deviation between different laboratories performing the same experiment, and contains additional sources of variation over the repeatability. A summary of the results obtained for the precision and trueness estimates are presented in **Table 6**.

Table 6. Summary of quantitative measurement data

	Sample nominal value (% horse meat w/w)				
Metric	**0.10**	**0.50**	**1.00**	**5.00**	**20.00**
Number of participating laboratories returning complete data sets	17	17	17	17	17
Number of laboratories removed from analysis*	4	4	4	4	4
Number of significant outliers determined with use of Grubbs outlier test	0	1**	0	0	0
Number of accepted laboratories	13	13	13	13	13
Relative repeatability standard deviation RSD_r (%)	15.00	8.39	8.65	6.66	5.74
Repeatability standard deviation S_r	0.02	0.05	0.11	0.41	1.34
Relative reproducibility standard deviation RSD_R (%)	25.83	17.58	13.73	13.35	12.35
Reproducibility standard deviation S_R	0.03	0.11	0.17	0.83	2.87
Assigned value based on sizes of equine and bovine genomes (% horse cp/cp)	0.11	0.55	1.10	5.48	21.58
Mean value based on collaborative trial (% horse cp/cp)	0.12	0.62	1.26	6.20	23.26
Bias (absolute) of the analytical data set	0.01	0.07	0.16	0.72	1.68
Bias (%) relative to assigned value based on genome sizes	9.00	12.68	14.55	13.19	7.76

** Data sets from four laboratories were removed from subsequent analysis as they failed to either meet accepted PCR performance criteria (**Annex 6**) (e.g. exhibiting poor PCR efficiency), or they reported technical issues with the instrument.*

*** A single test unit (U7) was removed from the 0.5% (w/w) data set for laboratory 10(F) as it was identified as a significant statistical outlier. All of the remaining data for this laboratory was retained. (For a detailed explanation please refer to section 5.5.1).*

The table reveals a general trend in the data where the repeatability and reproducibility improve (decrease) with increasing concentration. The reproducibility is below 26% across the range of DNA levels examined, while the repeatability is consistently 15% or below.

Since the original method for the relative quantitation of horse DNA was published in 2013 [4], recent advances in sequencing of mammalian genomes has resulted in estimates of the equine and bovine genomes becoming available. According to the NCBI database [18], the size of the equine genome can be estimated as 2474.94 M base pairs [19], and the bovine (cattle) genome as 2724.98 million base pairs [20]. These estimates suggest that the horse genome is around 17% smaller than the bovine genome. Based on these estimates, it would be prudent to take into account the relative genome size differences when calculating the assigned value of the test samples as otherwise bias may be introduced if the assigned values are based purely on the nominal w/w concentrations alone. **Table 6** shows the assigned value of the five sample levels, taking into account the relative genome size differences between the equine and bovine genome for the purposes of this study. The bias between the assigned value and the estimated value of each sample from the collaborative trial can then be calculated. There is a small but consistent positive bias in this data set, with estimated values varying between 8 and 15% depending upon the sample.

6. Discussion

The measurement criteria which are required to be satisfied in order that an experimental method be considered fit for purpose have been outlined in IUPAC and the ENGL guidelines [1,2]. Collectively, these require that: (a) the relative reproducibility standard deviation (RSD_R) should be below 35% over the majority of the dynamic range, and below 50% at the lower end of this range; (b) the relative repeatability standard deviation (RSD_r) should be below 25% across the levels of analyte tested.

As can be seen from the values summarised in **Table 6**, the real-time PCR method satisfies both of these requirements at all of the levels of horse DNA tested. Specifically, the highest value obtained for the RSD_R (%) was 25.83% at the 0.1% w/w level, which is below the critical threshold of 50% set for this lower level. With respect to the RSD_r it can be seen from **Table 6** that a maximum value of 15% was recorded for this metric at the 0.1% w/w level. This is below the critical threshold of 25% stipulated by the collective IUPAC and ENGL guidance.

Values for the trueness of the method can be estimated from the levels of bias observed at each of the levels of analyte used. According to IUPAC and ENGL guidelines, trueness should be ± 25% across the measured range. Taking into account the relative sizes of the equine and bovine genome based on recent DNA sequencing projects, the bias compared to the assigned value of the samples (on a copy number by copy number basis) varied between 7.76 and 14.55%, well within the ± 25% criteria for acceptance. The published guidance is also intended for use with well characterised reference materials and not necessarily for in-house materials which were prepared for use in this collaborative trial. The focus of this trial was to assess the performance of the method, and not to assign a value to the samples used. A relatively consistent and positive bias is shown throughout all sample levels, which is likely to have some contributions from the production of the

gravimetric samples. This is particularly relevant for the 0.1%, 0.5% and 1% w/w sample levels which were derived from an appropriate gravimetric amount of the 5% w/w material, and were thus not completely independent. To physically prepare materials at such low levels based on gravimetric preparations of the 100% materials alone was not feasible within the scope of this project, nor was it the sole focus of this project to accurately assign values to these samples. A consequence of this is that the 0.1%, 0.5% and 1% w/w samples are not totally independent of each other, and any bias introduced into the 5% material from which they were derived may also impact upon their true values. However, the estimates for repeatability, reproducibility and bias satisfy the combined requirements mentioned in the IUPAC and the ENGL guidance documents, providing evidence of the fitness for purpose of the method.

The focus of this study was to characterise the repeatability and reproducibility of the method as part of a collaborative trial, and not to assign a value to the five test samples. The debate whether to express results in terms of w/w or cp/cp, the advantages and disadvantages of the two, and the conversion between them, continues to be a focus of discussion at an international level between scientists, with no immediate agreement on a solution. Hence the “true” value of a test sample may never really be known. An interesting aspect to consider however, is the assignment of a value to the test samples used in this study based on the robust means taken from the collaborative trial. For each test sample, the robust mean was based on four biological replicates measured three times each across thirteen different laboratories. The very tight repeatability and reproducibility estimates associated with each of the five samples provide testament to the fact that there is reasonable confidence in the value that is being estimated.

In terms of the real-time PCR assays used with the method, both satisfy the required performance criteria specifically stipulated in the ENGL guidelines [2]. Specifically, mean PCR efficiencies of 94.1% and 96.4% were achieved by the horse specific (*EC-GHR1*) and universal mammalian (*MY*) assays respectively. These values correspond to a slope of the line of the standard curve of -3.5 and -3.4 respectively, which fall within the -3.1 ≥ slope ≥ -3.6 range stipulated in the ENGL guidelines [2]. In addition, the mean values for the correlation coefficient of a standard curve obtained by linear regression (R^2) were 0.998 and 0.997 for the horse specific (*EC-GHR1*) and universal mammalian (*MY*) assays respectively, which are above the threshold of acceptance stipulated in the ENGL guidelines [2]. A list of the method performance criteria as required by the ENGL has been included in **Annex 6**.

Recommendations

The repeatability and reproducibility estimates from the collaborative trial provide evidence for the good precision of the method within and between laboratories, and the bias associated with the estimated and assigned values of the test samples is well within acceptance criteria mentioned in the IUPAC and ENGL guidance. A main

recommendation from this project is that the method be considered further for progression towards standardisation at an ISO level.

The nature and format of the method follows experimental designs which are well established and accepted as common practice on an international basis, e.g. those followed for GMO analysis. However, whilst effective, such an experimental design uses an extensive calibration curve and utilises the two targets of the horse specific target and the mammalian target as separate singleplex assays. In order to make cost savings and accommodate more test samples within a batch run, it would be beneficial to consider possible routes to optimisation of the experimental design. Such options could include multiplexing the horse specific and mammalian targets together in the same assay, and/or using a delta delta C_q approach whereby a selection of controls are used instead of an extensive calibration curve. Both of these approaches would provide increased throughput and cost saving opportunities, and preliminary work using the delta delta C_q approach has provided encouraging results. It is a recommendation from this project that this approach be considered further, such that the delta delta C_q approach could be used as a rapid screening approach to identify problematic samples which would benefit from being analysed further using the full method described in this report.

Recent evidence from DNA sequencing projects has facilitated estimation of the sizes of the equine and bovine genomes. These estimates can be taken into account when specific conditions are met when assigning values to test samples based on w/w and copy number to copy number ratios, to facilitate more accurate comparisons. This was possible in the context of the current project because the methods' scope is designed to be used for samples consisting of horse meat in a beef (meat) background. From a metrological and traceable scientific point of view, estimates made in copy numbers are often preferable, but from a practical, enforcement and legislative view point, estimates based on w/w are often the preference. Copy number values of a sample can be affected by tissue type, DNA recovery, matrix background, DNA degradation, sample preparation etc., and it is not within the remit of the current project to resolve this situation, where discussions on the area continue to be the subject of many international working groups with no clear resolution to the issue. However, as estimates of mammalian genome sizes become increasing more accurate and available, for examples as a result of Next Generation Sequencing projects, it may become possible to take into account these calculations to provide more accurate estimates of sample levels on a copy number by copy number basis in controlled situations. Alternatively, digital PCR can be used to assign values to test samples based on absolute single molecule quantitation of the various mammalian genomes. It is therefore a recommendation of this project that mammalian genome size estimates be taken into account and modelled further, in order to provide more accurate value assignment of test samples on a copy number by copy number basis.

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Annexes

Contents

Annex 1: DNA extraction

DNA extraction methodologies can impact on the quantitative performance of a qPCR-based test through potential issues such as extraction carryover and DNA integrity. The SOP associated with the method as part of the international collaborative trial does not prescribe a particular extraction methodology as this would limit the applicability of the method. However, users should minimise the impact of the extraction methodology by using a single validated approach in the preparation of all DNA materials (test, control and calibrants). This is the same approach taken for validating new methods for GMO quantitation using real-time PCR, where pre-extracted DNA for evaluation is provided to participants as part of the method validation procedure (Mazzara *et al*., 2013) [17].

The SOP developers have evaluated a number of extraction methodologies and did not observe any significant analytical performance differences providing good laboratory practice is followed. A limited study comparing DNA extracts from two extraction approaches (an in-house CTAB-based method and a commercial Kleargene™ DNA extraction kit (LGC Ltd)) taken from the same 1% (w/w) gravimetric sample gave mean and 95% confidence intervals of 1.16 +/- 0.17 % and 1.31 +/- 0.31 % respectively, based on six independent replicates. The confidence intervals of the estimated values of the sample from the two extraction approaches overlap, providing evidence that estimated sample values should not be significantly different between DNA extraction approaches if quality procedures are adhered to.

Annex 2: DNA quality

Reliable measurement of DNA concentration and purity is important for many applications in molecular biology. The most common method of measuring DNA purity is by determining absorbance at specific wavelengths using a spectrophotometer. It is recommended that published quality criteria for extracted DNA (e.g. Mamiatis *et al*., 1985 [21]) should be implemented in order to verify the applicability of using extracted DNA for many down-stream applications, including real-time PCR. Examples of such criteria include: value for the A_{260}:A_{280} ratio, value for the A_{260}:A_{230} ratio, PCR efficiencies and calibration curve linearity.

Example guidance notes for determination of DNA purity

- The A_{260}:A_{280} for pure DNA should be close to 1.8. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants.
- The A_{260}:A_{230} should ideally be in the range of 2.0 to 2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm
- Gel based analysis should result in a broad brightly stained band positioned below the application well. Smears or comet tails are indicative of sample degradation and/or fragmentation. Similarly, off-set of Bioanalyzer profiles to the left are indicative of sample degradation and/or fragmentation

Annex 3: Standard operating procedure for the quantification of horse DNA relative to mammalian DNA in raw meat samples

DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

DRAFT STANDARD OPERATING PROCEDURE
HORSE RELATIVE QUANTITATION
Version 2.3, September 2015

DRAFT STANDARD OPERATING PROCEDURE FOR THE QUANTITATION OF HORSE DNA RELATIVE TO MAMMALIAN DNA IN RAW MEAT SAMPLES

Version 2.3

Prepared by: Gavin Nixon, Malcolm Burns, (LGC, Teddington)
Date: 24/09/15

Approved by: M. Burns
Date: 24/09/15

Contents

1. BACKGROUND

The EU and UK Horse meat issue in 2013, where a significant amount of horse DNA was found in a beef burger intended for sale to the public at a supermarket, brought into perspective the lack of harmonisation on how the amount of meat adulteration in a sample was expressed. As part of the response to the 2013 EU horse-meat issue, Defra/FSA commissioned an official UK Survey of beef products. This highlighted a requirement for a quantitative approach to be developed to accurately measure the amount of horse DNA present in samples, and led to a Defra funded project (FA0135) to develop a non-proprietary real-time PCR approach for the quantitation of horse DNA present in a test sample.

Horse meat content within the UK food chain does not typically represent a food safety problem, except for the potential presence of the restricted nonsteroidal anti-inflammatory drug phenylbutazone (bute). However, in order to support EU and UK Food law, it is necessary to be able to identify and quantify levels of meat food components (currently there is a recommended 1% threshold used as a guide to enforcement for use in relation to the presence of undeclared horse meat).

2. PURPOSE

The purpose of the method described within this Standard Operating Procedure is to quantify the amount of horse DNA relative to total mammalian DNA in raw meat samples, using real-time PCR. The method has been applied to and is validated for DNA extracted from samples that consist of raw horse meat in a raw beef (meat) background (raw muscle tissue rimmed free of surface inter-muscular fat and connective tissue).

3. SCOPE

The method described uses real-time PCR to quantitate the amount of horse DNA relative to the amount of total mammalian DNA extracted from a raw meat sample. The method has been applied to and is validated for DNA extracted from samples that consist of raw horse meat in a raw beef (meat) background. Results can be expressed relative to the amount of raw horse meat in a raw beef (meat) background on a gravimetric (w/w) basis.

4. DEFINITIONS AND ABBREVIATIONS

C_q – quantification cycle	R^2 - coefficient of determination
DNA – deoxyribonucleic acid	RQ – relative quantitation
qPCR – quantitative real-time PCR	w/w – weight for weight

5. PRINCIPLE OF THE METHOD

Test samples containing horse DNA in a background of beef DNA are analysed by a relative quantitation approach utilising singleplex qPCR assays targeting the horse

growth hormone receptor gene (Köppel *et al*., 2011) and the myostatin gene (Laube *et al*., 2003) present in mammals and poultry. DNA template concentration is quantified prior to the real-time PCR to normalise test input levels.

100% horse DNA derived from authenticated raw horse meat materials that have been extracted and treated in the same manner to the test samples should be used to generate separate calibration curves for both the horse specific target and the mammalian target based on estimated genome equivalents. Test samples are evaluated using the same horse specific and universal mammalian qPCR assays. Estimated genome equivalent copy numbers are determined for the test samples using the horse and mammalian calibration curves. The percentage horse DNA content of the test sample is expressed as a ratio of the number of horse genome equivalents relative to the total mammalian genome equivalents present in the sample.

Overview of method

DNA extracted from test sample and normalised to 50 ng per PCR well

↓

Test sample analysed by horse specific and mammalian universal qPCR assays

↓

C_q data converted to estimated genome equivalents by calibration curve approach (20,480 horse to 5 genome equivalent copies)

↓

Relative quantitation analysis performed, expressing the result as the percentage of horse to mammalian DNA

6. METHOD PERFORMANCE CHARACTERISTICS

6.1. Specificity

The specificity of the Köppel horse assay was confirmed experimentally as exhibiting no cross reactivity with beef, pork, lamb, duck, mouse, human and chicken DNA. The published literature states that the assay does not cross react with 50 other animal and plant species often found in food. It is known that the Köppel horse assay does cross react with DNA from mule/donkey. However, neither of these species are common meats used within the UK and their use as labelled ingredients is very unlikely. The undeclared presence of any meat species in a sample is considered non-compliant with EU labelling legislation.

The Laube assay targets the myostatin gene known to be present in mammalian, poultry and fish species. The assay was demonstrated experimentally to successfully amplify the target species and confirmed the specificity characteristics detailed within the published literature (DNA detected from eighteen mammalian and poultry species tested within the published study).

6.2. Limit of Detection (LOD)

The LOD was defined as the lowest target analyte concentration (estimated nominal copy numbers) that could still be detected on 95% of occasions. This

was determined experimentally to be less than 5 copies for both the horse genome and mammalian genome, based on raw meat samples.

6.3. Limit of Quantitation (LOQ)

The LOQ was defined as the lowest relative amount of horse content of a sample that could still be reliably quantified (95% CI incorporates assigned value). This was determined experimentally as <0.1% DNA:DNA ad-mixtures and as <0.1% w/w gravimetric materials of raw horse meat in a raw beef (meat) background.

7. SAFETY

7.1. National and laboratory safety procedures must be adhered to at all times. Analysts should consult laboratory COSHH assessments and MSDS prior to undertaking this method.

8. MATERIALS AND EQUIPMENT

8.1. Reagents: Unless otherwise stated, all reagents are of molecular biology grade quality. All reagents are sourced from reputable suppliers following appropriate quality systems.

8.1.1. 2x TaqMan Universal PCR Master Mix (Cat. No. 4304437, Life Technologies, Paisley, UK) containing qPCR buffer, hotstart taq polymerase and ROX passive reference dye.

8.1.2. Nuclease-free water (Cat. No. AM9937, Ambion brand, Life Technologies, Paisley, UK).

8.1.3. HPLC purity oligonucleotide primers and 6-FAM (reporter)/non-fluorescent quencher (NFQ) labelled hydrolysis probes (Table 1) sourced from Eurofins Genomics (Ebersberg, Germany).

Target	Assay details	Sequence Names	Sequence (5' – 3')	Labelling
Horse	Köppel *et al.* (2011)	EC-GHR1-F	CCAACTTCATCATGGACAACGC	
		EC-GHR1-R	GTTAAAGCTTGGCTCGACACG	
		EC-GHR1_(P)	AAGTGCATCCCCGTGGCCCCTCA	6-FAM/ NFQ
Mammalian & poultry myostatin gene	Laube *et al.* (2003)	MY-f	TTGTGCAAATCCTGAGACTCAT	
		MY-r	ATACCAGTGCCTGGGTTCAT	
		MY-Probe	CCCATGAAAGACGGTACAAGGTATACTG	6-FAM/ NFQ

Table 1. Primer and probe sequence information

8.2. Authenticated horse DNA sample: this must consist of raw muscle tissue trimmed free of surface inter-muscular fat and connective tissue. The source material should be derived from appropriate certified reference materials or in-house developed materials authenticated through appropriate meat speciation tests, e.g. qPCR and DNA sequencing-based analyses. Purified and quantitated genomic DNA derived from the authenticated 100% w/w horse muscle tissue must adhere to appropriate quality characteristics (i.e., A260/280 ~ 1.8, A260/230 ~ 1.8 to 2.2).

8.3. Test materials: Quantitated DNA extracts with appropriate quality characteristics (i.e., A260/A280 ~ 1.8, A260/A230 ~ 1.8 to 2.2).

8.4. A single validated DNA extraction methodology should be applied both to the authenticated meat reference materials and the test samples.

8.5. Equipment:

8.5.1. Standard laboratory personal protective equipment (PPE), e.g. safety glasses, latex/nitrile gloves and laboratory coat.

8.5.2. 10 % (v/v) Microsol 3+ (Cat. No. MIC-203, Anachem, Luton, UK) for decontaminating surfaces

8.5.3. Eppendorf Centrifuge 5424 bench top centrifuge for centrifuging plates/tubes.

8.5.4. Gilson Pipette PIPETMAN calibrated micropipettes (1 – 200 µl range).

8.5.5. Vortex mixer for sample/reagent mixing.

8.5.6. Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, Paisley, UK).

8.6. Consumables

8.6.1. MicroAmp® Optical 96-Well Reaction Plate with Barcode (Cat. No. 4306737), MicroAmp® Optical Adhesive Film (Cat. No. 4311971) and Applied Biosystems 7900HT Fast Real-Time PCR System (all sourced from Life Technologies, Paisley, UK).

8.6.2. Plastic micro tubes (for preparing reagents).

8.6.3. Micropipette tips in appropriate range.

9. PROCEDURE

9.1. This procedure was developed and validated on an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, Paisley, UK).

9.2. The procedure requires adherence to strict template (extracted DNA and amplicon) contamination control methodologies, i.e.

9.2.1. Laboratory organisation e.g. "flow direction" should follow international guidelines e.g. ISO 24276:2006.

9.2.2. Implement a spatial separation approach using dedicated equipment to minimise PCR contamination, i.e. separate area for assay preparation, template preparation, template addition and qPCR analyses.

9.2.3. Equipment and surfaces should be decontaminated with 10 % (v/v) Microsol 3+ to minimise template contamination.

9.3. Follow the instrument manufacturer's recommendations for setting up a real-time quantitative PCR experiment and adhere to MIQE guidelines (Bustin *et al.*, 2009) for recording and documenting experimental information/data.

9.4. Devise a 96-well plate design split between the Laube and Köppel qPCR assays comprising the following features:

9.4.1. Assessment of all samples individually by both a singleplex Laube (mammalian) assay and a singleplex Köppel (horse) qPCR assays.

9.4.2. Minimum triplicate PCR technical replication level to ensure data robustness.

9.4.3. Extraction control, PCR no template control (NTC) and positive control (50 ng horse DNA) to ensure data validity.

9.4.4. Horse DNA 7-point dilution series to be used as the calibrant (Section 9.7)

9.4.5. Test samples.

9.5. The method is developed for a total volume of 25 µl for the Laube (mammalian) reaction and 25 µl for the Köppel (horse) reaction. Combine 12.5 µl 2x TaqMan Universal PCR Master Mix with oligonucleotides (forward primer, reverse primer and probe to appropriate final reaction mixture concentration) and make up to 20 µl with DNase/DNA-free water (see Table 2). Scale reagent volumes to prepare sufficient reaction mixture for 1.2X number of reaction replicates. Transfer 20 µl of reaction mixture into the appropriate wells using a manual or automated micropipette.

Reagent Component	Laube Mammalian	Köppel Horse
2x TaqMan Universal PCR Master Mix	1x	1x
Forward Primer	0.3 µM	0.2 µM
Reverse Primer	0.3 µM	0.2 µM
Probe	0.2 µM	0.2 µM
Water	Make up to 20 µl	
Template DNA	5 µl volume Test samples: 50 ng DNA Standards: 111.1 ng to 0.03 ng DNA	

Table 2. qPCR reaction composition and final concentrations (25 µl total reaction volume)

9.6. DNA content of test samples should be quantified prior to dilution to ensure that 50 ng DNA test sample is added to each reaction well. Dilute test samples using DNase/DNA-free water.

9.7. Prepare a 7-point (4 fold) dilution series (S1-S7) ranging from approximately 20,480 horse to 5 genome equivalent copies, using quantitated 100% w/w horse genomic DNA as the calibrant, diluted to 22.22 ng μl^{-1} (111.10 ng per 5 µl) in DNase/DNA-free water (see Table 3).

Standard	DNA material (ng) per 5 µl	Genome equivalents per 5 µl	Volume of stock (µl)	Volume of diluent (µl)	Total volume (µl)*
S1	111.10	20,480	100.00	0.00	100.00
S2	27.78	5,120	25.00 (S1)	75.00	100.00
S3	6.94	1280	25.00 (S2)	75.00	100.00
S4	1.74	320	25.00 (S3)	75.00	100.00
S5	0.43	80	25.00 (S4)	75.00	100.00
S6	0.11	20	25.00 (S5)	75.00	100.00
S7	0.03	5	25.00 (S6)	75.00	100.00

Table 3. Preparation of a horse DNA 7 point, 4-fold calibration curve, based on initial 100% w/w horse DNA stock at 111.10 ng per 5 µl (equivalent to 20, 480 genome equivalents) and 100 µl dilution volumes. Genomic copy number estimations assume that 1 haploid copy equates to 2474.93 MB (NCBI Genomes, 2014). * Excluding dilution requirements.

9.8. Add test, calibrant and control samples (5 µl volume) to the MicroAmp® Optical 96-Well Reaction Plate and seal with MicroAmp® Optical Adhesive Film (all sourced from Life Technologies, Paisley, UK)

9.9. Centrifuge the plate at 1000g/1 min to ensure collection and to remove air bubbles.

9.10. Setup 'Standard Curve (AQ)' plate within the instrument SDS 2.4x software incorporating:

9.10.1. FAM/NFQ detector type per assay and appropriate sample types/names

9.10.2. Universal 2-step thermal cycling conditions with real-time data acquisition (see Table 4).

Temperature (°C)	Time (s)	Repeat Number
50	120	1
95	600	1
95 60	15 60	45

Table 4: PCR thermal cycling conditions

9.11. Automatic C_q determination performed unless data requires manual manipulation (as per instrument manufacturer's recommendations).

9.12. Export data from the SDS 2.4x software and analyse using Microsoft Excel (Microsoft UK, Reading, UK).

10. CALCULATIONS AND DATA ANALYSIS

10.1. See Appendix 2 for an example decision tree to support the testing procedure.

10.2. Generate horse-specific (Köppel) and mammalian universal (Laube) calibration curves (see Figure 1 for example plot):

10.2.1. Import the qPCR data generated by the SDS 2.4x software into Microsoft Excel (Microsoft UK, Reading, UK) and plot the $\log_{10}$ transformed estimated copy number (x-axis) versus mean C_q (y-axis) value for each of the seven standards used in the calibrant set.

10.2.2. Fit independent simple linear regression curves to the Köppel and Laube assay calibrant data sets to determine the equation of the straight line ($y = mx + c$) and coefficient of determination (R^2). (In Microsoft Excel 2010, this is achieved by highlighting the data points on the graph, right clicking with the mouse button, choosing "Add trend line . . ." from the drop down menu, choosing "Trendline options: linear" and checking the boxes for "Display Equation on Chart" and "Display R-squared value on chart)

10.2.3. Calculate % PCR efficiencies for both qPCR assays using the equation below wherein the "slope" is the gradient of the straight line (m):

$$\%\,\mathbf{E} = \mathbf{100} \times (\mathbf{-1} + \mathbf{10}^{\frac{-1}{slope}})$$

10.2.4. Performance metrics: R^2 should be above 0.98 and PCR efficiency 100% +/- 15% otherwise the experiment should be repeated.

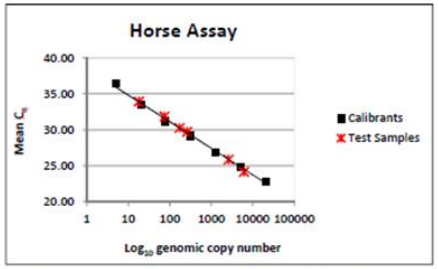


Figure 1. Example calibration curve for the horse specific (Köppel) assay. The $\log_{10}$ of the estimated copy number is plotted against the mean C_q value for the seven serial dilutions used in the calibration curve.

10.3. Calculate the percentage ratio of horse genome equivalents relative to the total mammalian genome equivalents present in the test sample:

10.3.1. Tabulate the mean C_q values for the horse specific (Köppel) and mammalian (Laube) assays for the test sample.

10.3.2. Using the previously derived equation of the straight line for both assays, rearrange the equations to calculate the estimated genomic DNA copy numbers for the horse and mammalian targets.

$$x = 10^{\frac{y-c}{m}}$$

x = estimated copy number
y = mean C_q value
c = intercept on the y-axis
m = gradient of the straight line

10.3.3. Calculate the estimated relative quantitative value of horse DNA compared to total mammalian DNA for each test sample using the following equation. This value provides an estimate of the horse content of a sample consisting of raw meat, relative to the amount of raw horse meat in a raw beef (meat) background on a gravimetric (w/w) basis.

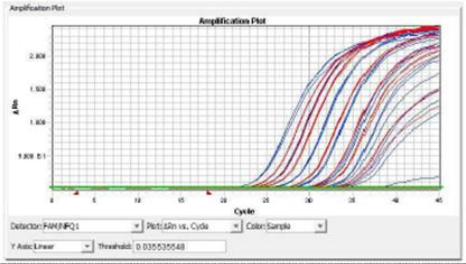
$$\%\ \text{horse meat} = \frac{\text{Calibration curve derived horse genome equivalent copy number}}{\text{Calibration curve derived total mammalian genome equivalent copy number}} \times 100$$

10.4. Please refer to Appendix 1 for a worked example.

11. APPENDICES

11.1. Appendix 1. Worked Example

Step	Details
1	**Collating qPCR data set**



- Apply the horse and mammalian qPCR assays to the test and calibrant samples to generate C_q values
- Export the C_q values to Microsoft Excel for analysis

2	**Generate calibration curves and associated quality metrics**

2a 1) Horse specific assay data

Sample Name	C_q	C_q Mean	C_q Std. Dev.
STD1	22.75	22.74	0.03
STD1	22.71		
STD1	22.76		
STD2	24.77	24.77	0.07
STD2	24.85		
STD2	24.70		
STD3	26.78	26.78	0.06
STD3	26.85		
STD3	26.72		
STD4	29.02	29.03	0.06
STD4	28.98		
STD4	29.09		
STD5	31.01	31.08	0.12
STD5	31.22		
STD5	31.02		
STD6	33.40	33.48	0.12
STD6	33.57		
STD6	33.46		
STD7	34.87	35.26	0.38
STD7	35.27		
STD7	35.64		
Test Sample	31.12	31.13	0.02
Test Sample	31.15		
Test Sample	31.11		

2) Mammalian assay data

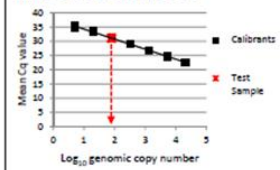
Sample Name	C_q	C_q Mean	C_q Std. Dev.
STD1	23.00	23.03	0.02
STD1	23.03		
STD1	23.06		
STD2	25.06	25.00	0.08
STD2	25.03		
STD2	24.90		
STD3	26.99	27.01	0.05
STD3	27.06		
STD3	26.98		
STD4	29.25	29.18	0.15
STD4	29.01		
STD4	29.27		
STD5	31.42	31.30	0.13
STD5	31.32		
STD5	31.16		
STD6	33.44	33.41	0.28
STD6	33.11		
STD6	33.67		
STD7	36.10	35.74	0.59
STD7	36.26		
STD7	35.87		
Test Sample	24.35	24.33	0.03
Test Sample	24.34		
Test Sample	24.30		

Calibrant Quantity (Nominal Copy No.)	20480	5120	1280	320	80	20	5
Log_{10} Quantity	4.31133	3.70927	3.10721	2.50515	1.90309	1.30103	0.69897

- Plot log_{10} transformed calibrant sample nominal copy number versus C_q value
- Produce separate calibration curves for both assays

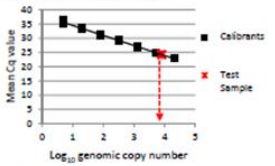
2b

1) Horse specific assay results



- Calibration curve metrics
  - m = -3.507
  - c = 37.793
  - R^2 = 0.998
- $\%\,E = 100 \times \left(-1 + 10^{\frac{-1}{-3.507}}\right) = 92.8\%$

2) Mammalian assay results



- Calibration curve metrics
  - m = -3.515
  - c = 38.044
  - R^2 = 0.997
- $\%\,E = 100 \times \left(-1 + 10^{\frac{-1}{-3.515}}\right) = 92.5\%$

- Apply linear regression to data set
  - $y = mx + c$
  - $y = C_q$, m = slope (gradient), x = log_{10} copy number, c = intercept on y-axis
- Determine calibration curve metrics
  - PCR efficiency (uses calibration curve slope)
  - R^2 (coefficient of determination)
- Determine PCR efficiency: $\%\,\mathbf{E} = \mathbf{100} \times \left(\mathbf{-1 + 10^{\frac{-1}{slope}}}\right)$
- Check that values fall within required range
  - R^2 >0.98
  - PCR efficiency 100% +/- 15%

3	**Copy number determination and calculate % horse adulteration**

3a

$$\mathbf{Copy\ number} = \mathbf{10^{\frac{C_q - intercept}{slope}}}$$

Test sample:

$$H_c = 10^{\frac{31.13 - 37.793}{-3.507}} = 79.42 \text{ copies}$$

$$M_c = 10^{\frac{24.33 - 38.044}{-3.515}} = 7971.95 \text{ copies}$$

- Calculate estimated horse (H_c)and mammalian (M_c) genomic copy number for each test sample using the rearranged equation of a straight line
- Collate data for all test samples

3b

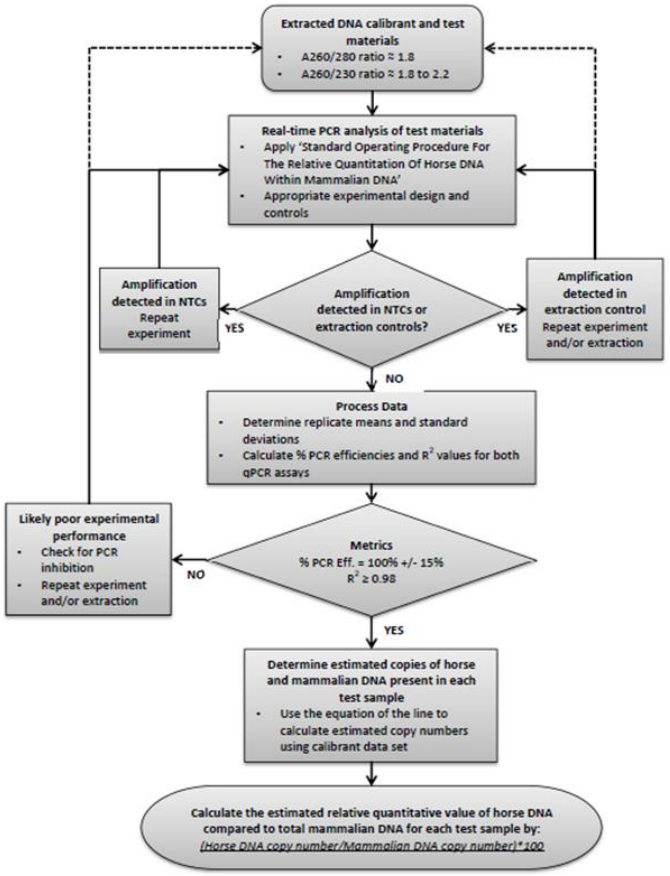
$$\%\ \mathbf{Horse\ adulteration\ content} = \frac{\boldsymbol{H_c}}{\boldsymbol{M_c}} \times \mathbf{100}$$

Test sample:

$$\%\ \text{Horse adulteration content} = \frac{79.42}{7971.95} \times 100 = 1.0\ \%$$

- Perform relative quantitative analysis to calculate % horse:mammalian DNA ratio

11.2. Appendix 2. Example Analytical Decision Tree



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Annex 4: Working instructions circulated to all participating laboratories in the collaborative trial

Collaborative Trial of a real-time PCR approach for the quantitation of horse DNA

This Collaborative Trial is organised by the United Kingdom Food Standards Agency (FSA), under the responsibility of the Molecular and Cell Biology Team of the Science and Innovation Division at LGC Ltd, Teddington (method developer).

General information

The protocol describes the application of a relative real-time PCR approach to quantitate the amount of (amplifiable) horse DNA relative to the amount of total (amplifiable) mammalian DNA extracted from a raw meat sample. The method has been applied to and validated for DNA extracted from samples that consist of raw horsemeat in a raw beef meat background. Results can be expressed relative to the amount of raw horsemeat in a raw beef meat background on a gravimetric weight for weight (w/w) basis.

The purpose of this trial is to use the protocol described below to estimate the relative horse DNA content of twenty test samples, labelled U1 to U20.

The PCR method has been optimised for use in an Applied Biosystems 7900HT Fast Real-time PCR instrument (Life Technologies, Paisley, UK). However, the methodology should be directly transferrable to any suitable real-time PCR instrument system compatible with the assay chemistry and experimental setup. In this collaborative trial, the method will be tested in a number of different laboratories in order to generate a comprehensive data set, which will be used to evaluate key performance parameters of the method. The aim of this comparative trial therefore is to test the performance of the method, and not that of a laboratory. Therefore, each laboratory should carefully follow the protocol provided. The subsequent statistical analysis of the results will be undertaken at LGC Teddington.

Materials provided to the participating laboratories

DNA test materials

The DNA test materials have been provided as single aliquots (incorporating an appropriate excess), with the exception of the calibrant, and should only be thawed prior to use. The test materials comprise the following:

A) Calibrant (C): Two tubes of calibrant are provided for use in the production of two standard curves (one tube per experimental run). One curve will be used with the myostatin (*MY*) assay (Laube *et al.* 2003) for the quantitation of total mammalian amplifiable DNA content. The second curve will be used with the equine growth hormone receptor (*EQ-GHR*) assay (Koppel *et al.*, 2011) for the quantitation of equine amplifiable DNA content. Each tube contains 210

µL of DNA solution, which is at a concentration of 26.05 ng/ µL and constitutes 3.5 times the amount that will be required in the analysis. The tubes should only be thawed as required.

B) Blind DNA test samples (U1–U20): These comprise of a mixture of DNA extracted from mixtures of raw horse meat in raw beef meat at varying levels. Twenty test samples (10 for each plate) are provided and are labelled U1 to U20. Each tube contains 105 µL of DNA solution. This represents 3.5 times the amount required in the analysis. The tubes should only be thawed as required.

All materials should be stored at -20°C (with the exception of the "TaqMan®Universal PCR Master Mix" which should be stored at +4°C immediately on receipt). After initial thawing, materials should be stored at 4°C for a maximum of 3 weeks.

Note: The PCR no-template control (NTC) (reaction mix + nucleic acid free water instead of DNA) must be prepared by the participant before each run.

Reagents Provided

A)Reaction Mix components

A) 2x TaqMan® Universal PCR Master Mix (Life Technologies, Part #4304437): One bottle containing 5 mL (Universal PCR Master Mix)

B)Primers and Probes

A) 25x *MY* (Universal mammalian) assay 168 µL

[NOTE: the *MY* probe is FAM labelled and BHQ1 quenched]

B) 25x *EQ-GHR1* (Equine specific) assay 168 µL

[NOTE: the *EQ-GHR1* probe is FAM labelled and BHQ1 quenched]

Materials and equipment required but not provided

- Optically compatible 96-Well reaction plates or other instrument specific reaction holder/s with minimum 96 reactions per run capacity
- Optical caps/adhesive covers
- Micropipettes
- Rack for reaction tubes
- 0.5 ml DNAse free reaction tubes
- 1.5 ml DNAse free reaction tubes
- Real-time PCR instrument and associated software
- Standard bench top centrifuge with rotor for 0.5 and 1.5 ml reaction tubes or standard microfuge
- Vortex mixer
- PCR-clean filter-tips
- Cold block or ice for setting up reactions
- Nucleic acid free water (for NTC)Protocol

Short description

The protocol is based on the Defra/LGC Standard Operating Procedure for the quantitation of horse DNA relative to mammalian DNA in raw meat samples, the original version can be found at the following link:

http://randd.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&ProjectID=19109&FromSearch=Y&Publisher=1&SearchText=FA0146&SortString=ProjectCode&SortOrder=Asc&Paging=10#Description.

The procedure follows the basic principles of the hydrolysis probe-based real-time PCR amplification method whereby the generation of specific PCR products are fluorescently monitored during each cycle (real-time) by means of a target-specific oligonucleotide probe.

Pairs of sequence-specific primers are employed to amplify a 107 bp fragment of the equine growth hormone receptor gene (*EC-GHR1*) in the case of the horse assay, and a 104-bp fragment of the myostatin gene (*MY*) in the case of the universal mammalian assay. Each assay also employs a sequence specific probe designed to be complementary to an internal region of the amplified fragment. Each of the probes possesses a 5' FAM reporter dye, and a 3' BHQ1 quencher. As the result of the inherent 5'-nuclease activity of the Taq DNA polymerase, cleavage of the probe removes the quencher (BHQ1) from the reporter dye (FAM), leading to an increased fluorescence that is then monitored.

The method employs a relative quantification approach that utilises the Standard Curve method. In each real-time PCR run, the C_q (also referred to as C_t) values are determined for all of the samples for both the horse specific *EC-GHR* , and universal mammalian *MY* assays in two separate but concurrent reactions (i.e. "singleplex" reactions) and using a triplicate level of replication. For ease of use, the 96-well plate is schematically divided in two parts (Figure 1.1 and 1.2), with the upper half of the plate (rows A to D) used for the amplification of the horse specific *EC-GHR1 assay,* and the lower half of the plate (rows E to H) used for the amplification of the universal mammalian *MY* assay.

For the quantification of horse DNA, two standard curves are produced, one with the horse specific *EC-GHR1 assay*, and one with the mammalian *MY* assay. Each standard curve consists of five calibration points that are produced using a 7 fold serial dilution of the DNA calibrant provided. The relative level of amplifiable horse DNA in each test sample is calculated as the ratio of *EC-GHR*/*MY* gene copy numbers as derived from the standard curve (relative quantification), and which can be expressed as a percentage for reporting purposes.

General instructions

- The procedure requires adherence to strict template (extracted DNA and amplicon) contamination control methodologies, i.e.
    - Maintain separate working areas with dedicated equipment and consumables for DNA handling, reaction set-up and amplification.

    - Use filter-plugged pipette tips in order to avoid cross-contamination.
    - Use only powder-free gloves and change them frequently.
    - Clean lab-benches and equipment periodically with 10% sodium hypochlorite solution or equivalent nucleic acid decontamination solution.
- All pipettes should be checked regularly for precision and, if necessary, should be calibrated.
- Strictly follow the indications on preparation of the reaction mixes and samples. These indications are of extreme importance to minimise variation resulting from pipetting.
- The plate layout provided should be followed by all participants, with the exception of non-plate based platforms where in the plate layout should guide the set-up process.

Real-time PCR approach for the quantitation of horse DNA– Operative protocol

#IMPORTANT NOTE: Although a large excess of reagents and samples is provided, please perform only the experiments specifically required, i.e. two quantification runs. Only the results of the two quantification runs should be provided to trial coordinator (LGC). Please use the excess material to repeat a run only in the case of a major technical problem (i.e. erroneous preparation of reaction mix, power failure, general amplification failure etc.).

Two sets of 96 reactions each have to be run. Each qPCR run is split into universal mammalian reference gene (*MY*), and horse specific (*EC-GHR*) reactions. Please strictly follow the prescribed plate layout.

1. Thaw, mix and centrifuge the components needed for the run. **Keep thawed reagents on ice**.
2. In two 1.5 mL tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the equine specific *assay* (Table 1) and the universal mammalian *MY* reference gene *assay* (Table 2). Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.
3. Mix well and centrifuge briefly.
4. Aliquot 20 µL of the universal mammalian *MY assay* reaction mix into each well of rows E - H and 20 µl of the horse specific *EC*-GHR *assay* reaction mix into each well of rows A - D according to the Plate Setup (Figure 1).
5. Seal the plate with a temporary optical cover and centrifuge the plate at 1000g/1 min to ensure collection and to remove air bubbles. Place on ice in the dark until required.

6. Prepare a 5 point (7 fold) dilution series (S1-S5) ranging from 24,010 to 10 horse genome equivalent copies per reaction using the calibrant provided (see Table 3). It is recommended that this be performed by employing an initial volume of 50 µL of the stock calibrant (S1), and transferring a 7 µL aliquot of this to 42 µL of an appropriate diluent (nuclease free water) to generate the second point in the series (S2). The dilution (S2) should be mixed thoroughly before transferring a 7 µL aliquot of the diluted stock (S2) to 42 µL of diluent to generate the third dilution in the series (S3). The process should then be repeated in order to generate the fourth (S4) and fifth (S5) dilution points in the series.
7. Add samples in order of NTC, standard curves, test samples (5 µL volume) to the reaction plate according to the layout specified in the plate layout scheme (Figure 1.1and 1.2).
8. Seal the plate accordingly and centrifuge at 1000g/1 min to ensure collection and to remove air bubbles.
9. Place the reaction plate in the real-time PCR instrument (possibly apply a compression pad, depending on the model) and start the run.
10. Programme the real-time PCR instrument according to the manufacturer's instructions and your Standard Operating Procedures.
    - Select FAM as reporter dye for both the equine specific and universal mammalian *assay*.
    - Define BHQ1 as the non-fluorescent quencher for both the equine specific (*EC-GHR*) and universal mammalian (*MY*) *assays*.
    - Enter the correct reaction volume (25 µL).
    - Cycle the samples as described in Table 4.

Table 1. Preparation of the reaction mix for one qPCR run of the horse specific *EC-GHR* assay

Component	Final concentration	Volume/ reaction (µL)	Total volume (µL) (53 reactions)
2x TaqMan Universal PCR Master Mix	1 x	12.5	662.5
25x equine specific EC-GHR assay, primer and probe mix	1 x	1.0	53.00
Nuclease Free Water		6.5	344.5
(DNA)		(5.0)	
Total volume		25.00	1060.00

Table 2. Preparation of the reaction mix for one qPCR run of the universal mammalian *MY assay*

Component	Final concentration	Volume/ reaction (µL)	Total volume (µL) (53 reactions)
2x TaqMan Universal PCR Master Mix	1 x	12.5	662.5
25x universal mammalian /poultry *MY* assay, primer and probe mix	1 x	1.0	53.00
Nuclease Free Water		6.5	344.5
(DNA)		(5.0)	
Total volume		25.00	1060.00

Table 3. Preparation of a horse DNA 5 point, 7-fold calibration curve, based on initial 100% w/w horse DNA stock at 26.05 ng/µL

Standard	DNA material (ng) per 5 µL	Genome equivalents per 5 µL	Volume of stock (µL)	Volume of diluent (µL)	Total volume (µL)
S1	130.25	24010.1	50	0.00	50
S2	18.61	3430.54	7	42	49
S3	2.66	490.34	7	42	49
S4	0.38	70.05	7	42	49
S5	0.05	9.21	7	42	49

11. *Set the real-time PCR standard mode, if applicable on your real-time PCR equipment*
12. **Notes for the labelling of the samples**:
    - Define the single wells as independent samples and not as replicates (leave the replicate field empty).
    - All test samples can be defined as "unknown".
    - Please use the following codes for samples:
        - U1 – U20 = test samples U1 - U20 each in three replicates
        - S1 – S5 = Standard curve samples S1 - S5 each in three replicates
        - NTC = PCR No Template Control (nucleic acid free water).

Table 4. Cycling program for the horse specific (*GHR*) & universal mammalian (*MY*) assays.

Step	Stage	Temperature °C	Time (sec)	Acquisition	Repeat Number
1	UNG activation	50	120	No	1x
2	Initial denaturation	95	600	No	1x
3	Denaturation	95	15	No	45x
	Annealing and extension	60	60	Yes	

Plate setup – loading order

Two plates (A and B) should be run, on each of which both the universal mammalian reference gene sequence (*MY*) and the horse specific gene sequence (*EC-GHR*) are amplified..

Figure 1.1. Plate set-up and loading order for Plate A (Samples U1 – U10)

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
B	STD5	STD5	STD5	NTC	NTC	NTC	U1	U1	U1	U2	U2	U2
C	U3	U3	U3	U4	U4	U4	U5	U5	U5	U6	U6	U6
D	U7	U7	U7	U8	U8	U8	U9	U9	U9	U10	U10	U10

Upper half: horse specific (EC-GHR) assay

Lower half: universal mammalian (MY) assay

	1	2	3	4	5	6	7	8	9	10	11	12
E	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
F	STD5	STD5	STD5	NTC	NTC	NTC	U1	U1	U1	U2	U2	U2
G	U3	U3	U3	U4	U4	U4	U5	U5	U5	U6	U6	U6
H	U7	U7	U7	U8	U8	U8	U9	U9	U9	U10	U10	U10

- U1 – U10 = test samples U1 – U10 each in three replicates
- S1 – S5 = Standard curve samples S1 - S5 each in three replicates
- NTC = PCR No Template Control (nucleic acid free water).

Figure 1.2. Plate set-up and loading order for Plate B (Samples U11 – U20)

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
B	STD5	STD5	STD5	NTC	NTC	NTC	U11	U11	U11	U12	U12	U12
C	U13	U13	U13	U14	U14	U14	U15	U15	U15	U16	U16	U16
D	U17	U17	U17	U18	U18	U18	U19	U19	U19	U20	U20	U20

Upper half: horse specific (EC-GHR) assay

Lower half: universal mammalian (MY) assay

	1	2	3	4	5	6	7	8	9	10	11	12
E	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
F	STD5	STD5	STD5	NTC	NTC	NTC	U11	U11	U11	U12	U12	U12
G	U13	U13	U13	U14	U14	U14	U15	U15	U15	U16	U16	U16
H	U17	U17	U17	U18	U18	U18	U19	U19	U19	U20	U20	U20

- U11 – U20 = test samples U11 - U20 each in three replicates
- S1 – S5 = Standard curve samples S1 - S5 each in three replicates
- NTC = PCR No Template Control (nucleic acid free water).

Data processing

Important preliminary notes:

- Use the sample labelling as described in the above procedure.
- Use the values of all the replicates for the standard curve (and not their mean value).
- Eliminate only obvious outliers from the calculation of the calibration curves and test samples. However, the submitted results must include the original unedited dataset.

Following the real-time PCR run, copy (copy-and-paste) all raw data to the correct Excel worksheet of the "LABxx-Horse_Collaborative_Trial _Results.xls" file provided. Use the worksheet named "Raw_Data_PLATE_A" to record the Plate A run data, and the worksheet named "Raw_Data_PLATE_B" for the Plate B run data.

The sheets labelled "Analysis_PLATE_A" and "Analysis_PLATE_B" should be used to perform the relative quantitation for equine DNA analyses for all of the test samples by pasting the relevant data into the template provided. The formulae embedded in the workbook will automatically calculate the parameters associated with the Standard Curve (slope, intercept, R^2) and the % horse DNA content for each of the test samples.

Documentation of results

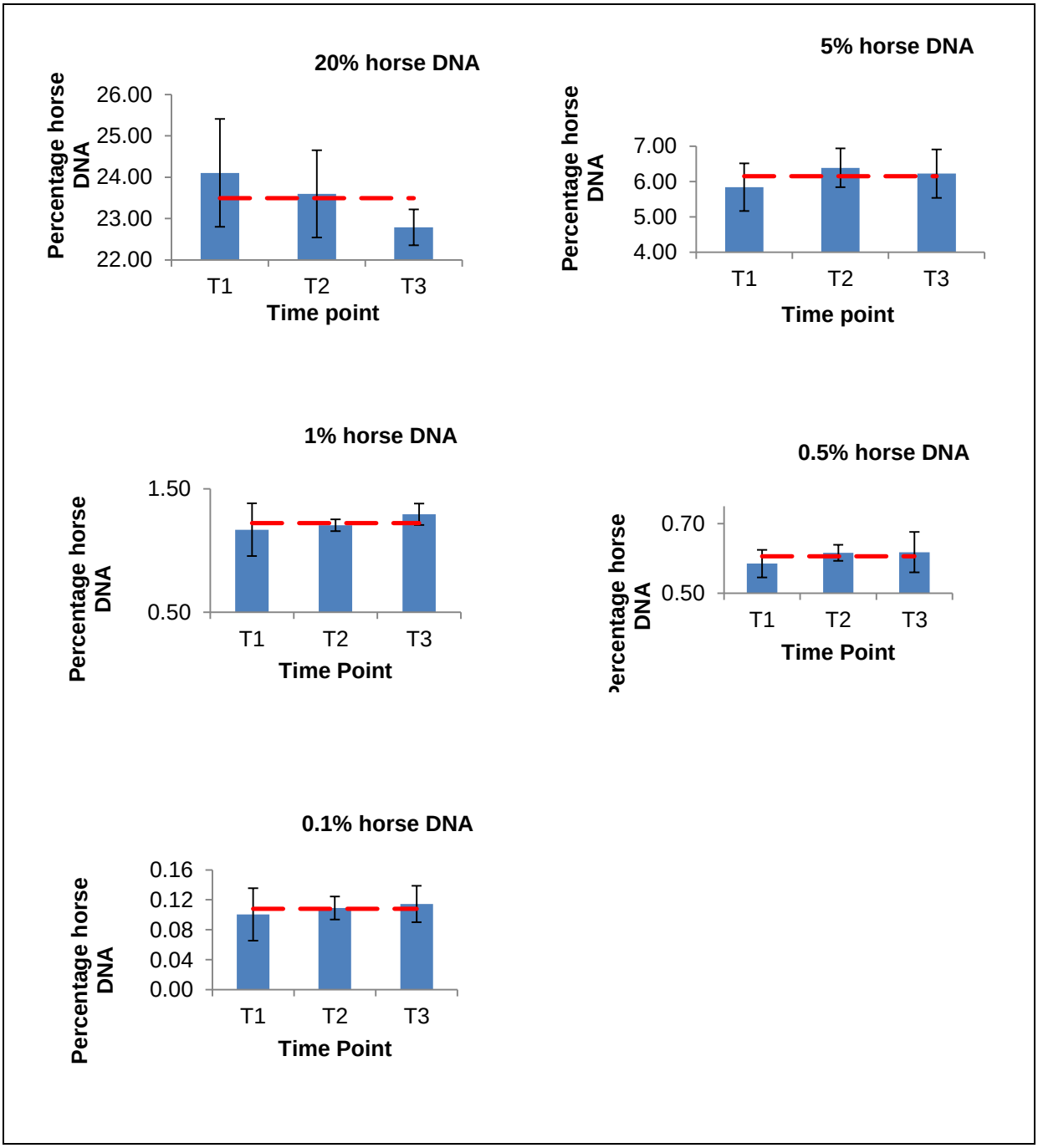
Please complete the "LABxx-Horse_Collaboarative_Trial_Results" file so that it contains the raw and analysed data from Plates A and B, the % Horse DNA calculations of all test samples and completed laboratory detail record.

Please record any deviations from the Comparative Trial protocol and any comments on how we can improve the Trial in the "Laboratory Details" worksheet.

Please send the files by email to the following address:
Timothy.Wilkes@iqcgroup.com

Annex 5: Limited stability and homogeneity study

Figure 1. Mean quantitative values obtained for percentage horse DNA present in test samples as measured at three separate time points (T1, T2 and T3). Overall mean for each of the levels indicated by the broken red line. Error bars represent one standard deviation either side of the mean.



Annex 6: Minimum Performance Requirements as set by the European Network of GMO Laboratories (ENGL)

In order for a method to be considered as fit for purpose, the performance requirements listed below are required to be fulfilled during an evaluation of a real-time PCR assay through a collaborative trial, as described in the Minimum Performance Requirements for Analytical Methods of GMO Testing [2]. The list below includes both the definition of, and acceptance criteria for each of the performance requirements.

- ***Repeatability Standard Deviation (RSD_r)***
  - Definition: The relative standard deviation of the test results obtained under repeatability conditions. Repeatability conditions are those where the test result has been obtained with the same method, on identical test samples, in the same laboratory, by the same operator, using the same equipment within a short interval of time.
  - Acceptance criteria: The relative repeatability standard deviation should be ≤ 25% over the whole dynamic range of the method. Estimates of repeatability are recommended to be obtained for a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).
- ***Reproducibility Standard Deviation (RSD_R)***
  - Definition: The relative standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment.
  - Acceptance criteria: The relative reproducibility standard deviation should be below 35% over the whole dynamic range of the method. An RSD_R of < 50% is considered acceptable for concentrations below 0.2%.
- **Amplification Efficiency** (associated with the PCR)
  - Definition: The rate of amplification that leads to a theoretical slope of -3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated from the following equation. Efficiency =$[10^{(-1/slope)}]$ -1.
  - Acceptance criteria: The average value of the slope of the standard curve shall be in the range of (-3.1 ≥ slope ≥ -3.6).
- **R^2 Coefficient** (associated with the PCR)
  - Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.
  - Acceptance criteria: The average R^2 value should be ≥ 0.98.
- **Trueness**
  - Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias
  - Acceptance criteria: Trueness shall be within ± 25% of the accepted reference value over the whole dynamic range.

Annex 7: Summary of the PCR performance characteristics including, data linearity (R^2), slope and PCR efficiency (%)

The values presented in **Table 1** and **Table 2** represent an estimation of data linearity (R^2), slope of the line of best fit, and associated PCR efficiency for the horse specific and universal mammalian real-time PCR assays respectively.

Table 1: PCR performance metrics returned by each laboratory for the horse specific (EC-GHR1) real-time PCR assay.

Lab	Plate	Linearity (R^2)	Slope	PCR Efficiency (%)
A	1	0.997	-3.260	102.645
	2	0.997	-3.519	92.376
B	1	0.997	-3.511	92.679
	2	0.997	-3.466	94.324
C	1	0.999	-3.479	93.849
	2	0.999	-3.561	90.896
D	1	0.999	-3.472	94.104
	2	1.000	-3.267	102.349
E	1	1.000	-3.429	95.718
	2	0.998	-3.612	89.171
F	1	0.998	-3.628	88.640
	2	0.997	-3.623	88.822
G	1	1.000	-3.505	92.892
	2	0.999	-3.439	95.328
H	1	0.999	-3.351	98.785
	2	0.998	-3.449	94.942
I	1	0.998	-3.553	91.184
	2	0.999	-3.351	98.801
J	1	0.999	-3.528	92.058
	2	1.000	-3.559	90.977
K	1	0.993	-3.587	90.014
	2	0.998	-3.489	93.438
L	1	1.000	-3.450	94.916
	2	1.000	-3.432	95.607
M	1	0.999	-3.411	95.718
	2	1.000	-3.441	95.256
	Mean	**0.998**	**-3.476**	**94.057**

Table 2: PCR performance metrics returned by each laboratory for the universal mammalian (*MY*) real-time PCR assay.

Lab	Plate	Linearity ($R^{2)}$	Slope	PCR Efficiency (%)
A	1	0.999	-3.388	97.290
	2	0.998	-3.388	97.297
B	1	0.996	-3.405	96.045
	2	0.992	-3.050	112.765
C	1	0.987	-3.563	90.837
	2	0.999	-3.678	87.013
D	1	0.998	-3.579	90.461
	2	0.997	-3.357	98.557
E	1	0.999	-3.473	94.060
	2	1.000	-3.424	95.910
F	1	0.999	-3.489	93.460
	2	1.000	-3.340	99.250
G	1	0.999	-3.620	88.895
	2	0.999	-3.417	96.164
H	1	1.000	-3.263	102.537
	2	1.000	-3.285	101.569
I	1	0.990	-3.380	97.350
	2	0.998	-3.492	93.350
J	1	0.999	-3.414	96.288
	2	0.999	-3.426	95.847
K	1	0.994	-3.530	91.986
	2	0.998	-3.471	94.119
L	1	0.999	-3.224	104.269
	2	1.000	-3.257	102.774
M	1	0.998	-3.365	98.254
	2	0.999	-3.600	89.584
	Mean	**0.997**	**-3.418**	**96.382**

Annex 8: Summary of the collated quantitative data for the International Collaborative Trial of the real-time PCR method for the quantitation of horse DNA

The collated data for the percentage horse DNA present in the blind labelled test units (U1 to U20) provided as part of the collaborative trial, and as determined by participating laboratories with use of the real-time PCR method for the quantitation of horse DNA (**Table 1)**. The data presented represent the result for the 13 laboratories who returned their results and which passed the quality screening procedure implemented.

Table 1: The collated quantitative data set which was used for the analysis of the real-time PCR method's performance

Lab	Level % (w/w)																			
	0.10				0.50				1.00				5.00				20.00			
	U3	U8	U13	U17	U5	U10	U11	U15	U1	U9	U16	U19	U4	U7	U14	U18	U2	U6	U12	U20
A	0.07	0.07	0.14	0.10	0.41	0.39	0.67	0.59	1.02	1.01	1.23	1.00	5.24	5.00	5.80	5.64	21.27	21.49	22.52	21.13
B	0.09	0.09	0.14	0.09	0.52	0.67	0.70	0.59	1.15	1.01	1.40	1.26	4.70	4.83	4.40	5.52	17.23	13.99	28.29	26.71
C	0.14	0.12	0.10	0.15	0.64	0.68	0.71	0.56	1.40	1.15	1.02	1.18	6.69	6.73	5.33	6.44	21.50	23.10	18.84	23.74
D	0.19	0.16	0.08	0.09	0.91	0.75	0.56	0.50	1.50	1.64	1.14	1.19	8.06	6.93	5.92	6.07	31.80	30.04	26.04	21.92
E	0.10	0.08	0.13	0.15	0.57	0.56	0.74	0.68	1.32	1.31	1.60	1.62	6.71	6.90	6.55	7.34	24.14	26.00	26.16	26.13
F	0.17	0.16	0.14	0.12	0.61	0.65	0.57	0.55	1.44	1.36	1.16	1.32	6.27	**16.92***	6.57	5.78	25.10	22.98	21.19	20.71
G	0.10	0.07	0.10	0.09	0.44	0.50	0.56	0.51	1.14	1.21	1.05	1.07	5.49	6.20	5.70	6.33	23.10	20.58	22.71	21.13
H	0.08	0.09	0.11	0.11	0.53	0.51	0.57	0.56	0.96	1.09	1.21	1.19	5.37	4.98	6.18	6.09	21.30	21.52	22.80	21.92
I	0.12	0.10	0.13	0.12	0.59	0.64	0.48	0.54	1.27	1.42	1.27	1.20	6.44	6.35	5.50	6.14	24.36	22.43	23.77	21.78
J	0.12	0.12	0.13	0.17	0.72	0.58	0.71	0.62	1.22	1.35	1.42	1.35	6.45	6.52	6.33	6.56	24.38	24.91	24.82	26.33
K	0.14	0.17	0.18	0.15	0.81	0.83	0.75	0.82	1.25	1.72	1.35	1.35	7.73	8.15	7.51	7.42	23.17	24.58	24.67	24.76
L	0.12	0.15	0.12	0.13	0.65	0.66	0.66	0.58	1.16	1.28	1.07	1.24	6.16	6.34	6.23	5.84	21.96	22.13	23.19	24.00
M	0.11	0.08	0.13	0.13	0.59	0.60	0.70	0.57	1.22	1.32	1.40	1.21	5.32	6.35	6.98	6.31	22.86	23.35	22.44	22.50

** A single data point of 16.92 (Lab F, Test unit U7) was identified as a statistical outlier and removed from subsequent analysis of the raw data. All remaining data from Lab F was retained.*