DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

STANDARD OPERATING PROCEDURE HORSE RELATIVE QUANTITATION Version 2.1, March 2015

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

Version 2.1

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Date: 11/03/15

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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 recommendations and UK Food Labelling Regulations, it is necessary to be able to identify and quantify levels of meat food components in relation to labelling thresholds (currently there is a 1% w/w labelling threshold advocated for use in relation to the presence of 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

DNA – deoxyribonucleic acid

qPCR – quantitative real-time PCR

R^2 - coefficient of determination

RQ – relative quantitation

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 (Koppel *et al*., 2011) and the myostatin gene (Laube *et al*., 2003) present in mammals, poultry and fish. 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 Koppel 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 Koppel 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	Koppel *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 Koppel qPCR assays comprising the following features:

9.4.1. Assessment of all samples individually by both a singleplex Laube (mammalian) assay and a singleplex Koppel (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 Koppel (horse) reaction. Combine 12 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	Koppel 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 (^{o}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 (Koppel) 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 Koppel 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):

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

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

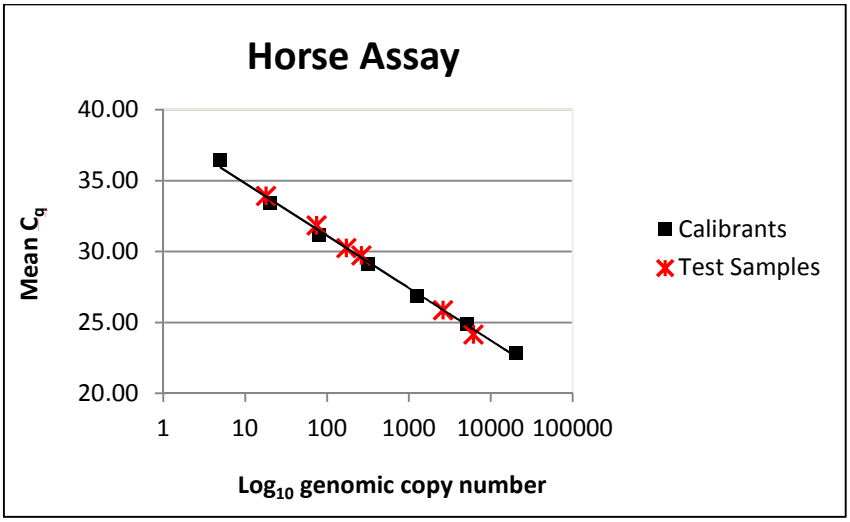


Figure 1. Example calibration curve for the horse specific (Koppel) assay. The $\log_{10}$ of the estimated copy number is plotted against the mean C_q value for the seven serial dilutions used in the calibration curve.

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 (Koppel) 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

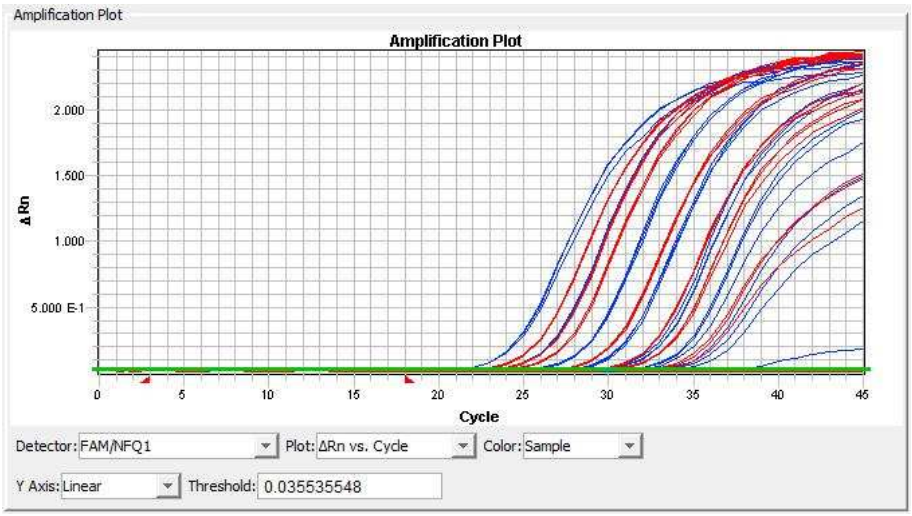
10.3.3. Calculate the estimated relative quantitative value of horse DNA compared to total mammalian DNA for each test sample using the following equation. This value provides an estimate of the horse content of a sample consisting of raw meat, relative to the amount of raw horse meat in a raw beef (meat)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

<table>
<tr><th>Step</th><th>Details</th></tr>
<tr><td>1</td><td>Collating qPCR data set</td></tr>
<tr><td></td><td> 
</td></tr>
<tr><td></td><td>• 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</td></tr>
<tr><td>2</td><td>Generate calibration curves and associated quality metrics</td></tr>
<tr><td>2a</td><td>1) Horse specific assay data
<table>
<tr><th>Sample Name</th><th>C_q</th><th>C_q Mean</th><th>C_q Std. Dev.</th></tr>
<tr><td>STD1</td><td>22.75</td><td>22.74</td><td>0.03</td></tr>
<tr><td>STD1</td><td>22.71</td><td></td><td></td></tr>
<tr><td>STD1</td><td>22.76</td><td></td><td></td></tr>
<tr><td>STD2</td><td>24.77</td><td>24.77</td><td>0.07</td></tr>
<tr><td>STD2</td><td>24.85</td><td></td><td></td></tr>
<tr><td>STD2</td><td>24.70</td><td></td><td></td></tr>
<tr><td>STD3</td><td>26.78</td><td>26.78</td><td>0.06</td></tr>
<tr><td>STD3</td><td>26.85</td><td></td><td></td></tr>
<tr><td>STD3</td><td>26.72</td><td></td><td></td></tr>
<tr><td>STD4</td><td>29.02</td><td>29.03</td><td>0.05</td></tr>
<tr><td>STD4</td><td>28.98</td><td></td><td></td></tr>
<tr><td>STD4</td><td>29.09</td><td></td><td></td></tr>
<tr><td>STD5</td><td>31.01</td><td>31.08</td><td>0.12</td></tr>
<tr><td>STD5</td><td>31.22</td><td></td><td></td></tr>
<tr><td>STD5</td><td>31.02</td><td></td><td></td></tr>
<tr><td>STD6</td><td>33.40</td><td>33.48</td><td>0.12</td></tr>
<tr><td>STD6</td><td>33.57</td><td></td><td></td></tr>
<tr><td>STD7</td><td>33.46</td><td></td><td></td></tr>
<tr><td>STD7</td><td>34.87</td><td>35.26</td><td>0.38</td></tr>
<tr><td>STD7</td><td>35.27</td><td></td><td></td></tr>
<tr><td>STD7</td><td>35.64</td><td></td><td></td></tr>
<tr><td>Test Sample</td><td>31.12</td><td>31.13</td><td>0.02</td></tr>
<tr><td>Test Sample</td><td>31.15</td><td></td><td></td></tr>
<tr><td>Test Sample</td><td>31.11</td><td></td><td></td></tr>
</table>
2) Mammalian assay data
<table>
<tr><th>Sample Name</th><th>C_q</th><th>C_q Mean</th><th>C_q Std. Dev.</th></tr>
<tr><td>STD1</td><td>23.00</td><td>23.03</td><td>0.02</td></tr>
<tr><td>STD1</td><td>23.03</td><td></td><td></td></tr>
<tr><td>STD1</td><td>23.05</td><td></td><td></td></tr>
<tr><td>STD2</td><td>25.06</td><td>25.00</td><td>0.08</td></tr>
<tr><td>STD2</td><td>25.03</td><td></td><td></td></tr>
<tr><td>STD2</td><td>24.90</td><td></td><td></td></tr>
<tr><td>STD3</td><td>26.99</td><td>27.01</td><td>0.05</td></tr>
<tr><td>STD3</td><td>27.06</td><td></td><td></td></tr>
<tr><td>STD3</td><td>26.98</td><td></td><td></td></tr>
<tr><td>STD4</td><td>29.25</td><td>29.18</td><td>0.15</td></tr>
<tr><td>STD4</td><td>29.01</td><td></td><td></td></tr>
<tr><td>STD4</td><td>29.27</td><td></td><td></td></tr>
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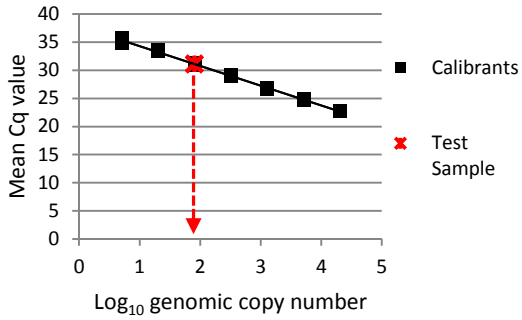
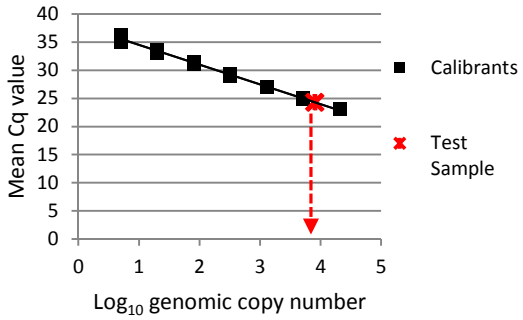
<tr><td>STD5</td><td>31.32</td><td></td><td></td></tr>
<tr><td>STD5</td><td>31.16</td><td></td><td></td></tr>
<tr><td>STD6</td><td>33.44</td><td>33.41</td><td>0.28</td></tr>
<tr><td>STD6</td><td>33.11</td><td></td><td></td></tr>
<tr><td>STD6</td><td>33.67</td><td></td><td></td></tr>
<tr><td>STD7</td><td>35.10</td><td>35.74</td><td>0.59</td></tr>
<tr><td>STD7</td><td>36.26</td><td></td><td></td></tr>
<tr><td>STD7</td><td>35.87</td><td></td><td></td></tr>
<tr><td>Test Sample</td><td>24.35</td><td>24.33</td><td>0.03</td></tr>
<tr><td>Test Sample</td><td>24.34</td><td></td><td></td></tr>
<tr><td>Test Sample</td><td>24.30</td><td></td><td></td></tr>
</table>
<table>
<tr><td>Calibrant Quantity (Nominal Copy No.)</td><td>20480</td><td>5120</td><td>1280</td><td>320</td><td>80</td><td>20</td><td>5</td></tr>
<tr><td>Log_{10} Quantity</td><td>4.31133</td><td>3.70927</td><td>3.10721</td><td>2.50515</td><td>1.90309</td><td>1.30103</td><td>0.69897</td></tr>
</table></td></tr>
<tr><td></td><td>• Plot log_{10} transformed calibrant sample nominal copy number versus C_q value
• Produce separate calibration curves for both assays</td></tr>
</table>

<table>
<tr><td>2b</td><td>
1) Horse specific assay results


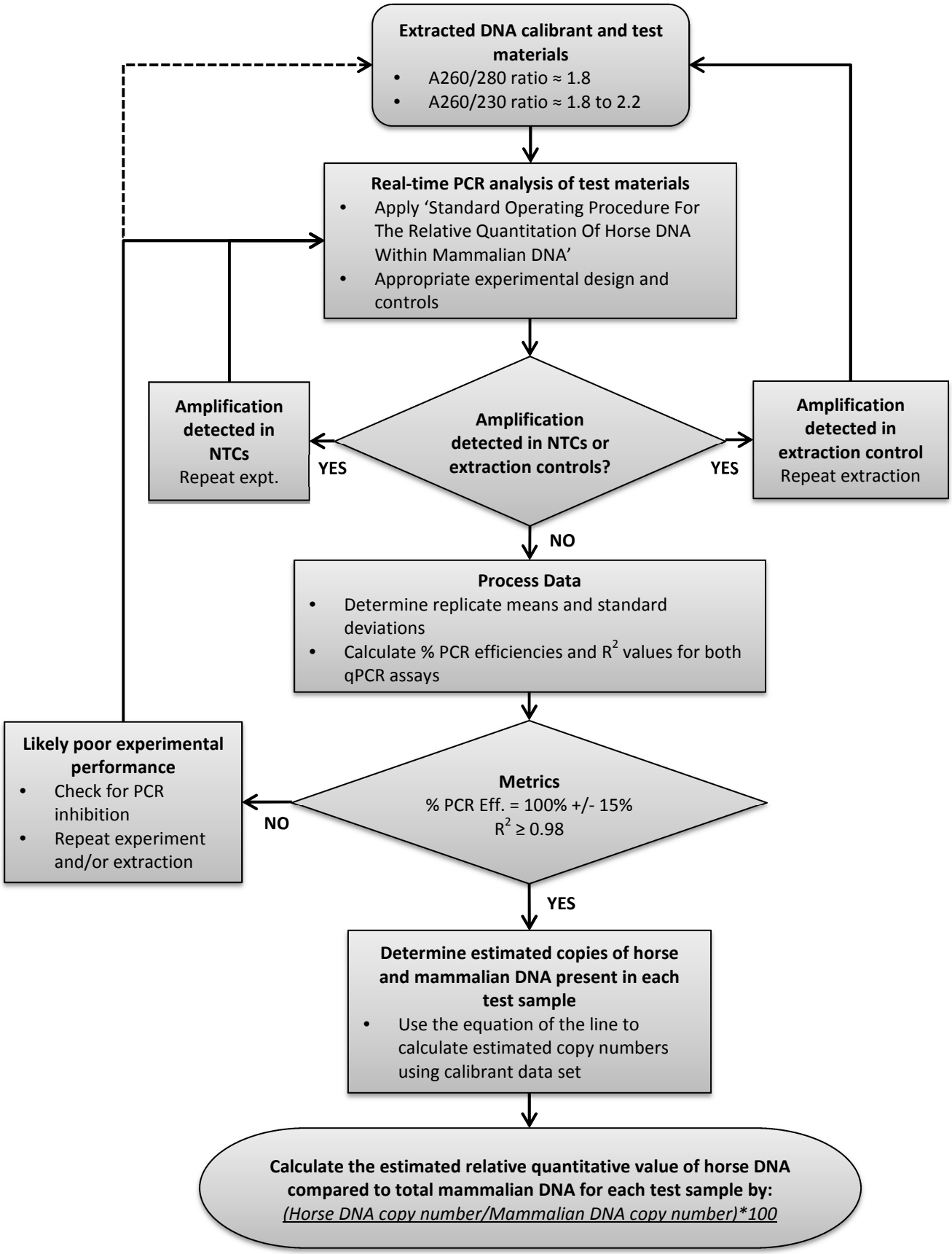
• Calibration curve metrics
o m = -3.507
o c = 37.793
o R^2 = 0.998
• $\%\,\mathrm{E} = 100 \times \left(-1 + 10^{\frac{-1}{-3.507}}\right) = 92.8\%$
2) Mammalian assay results


• Calibration curve metrics
o m = -3.515
o c = 38.044
o R^2 = 0.997
• $\%\,\mathrm{E} = 100 \times \left(-1 + 10^{\frac{-1}{-3.515}}\right) = 92.5\%$
</td></tr>
<tr><td></td><td>
• Apply linear regression to data set
o y = mx + c
o y = C_q, m = slope (gradient), x = $\log_{10}$ copy number, c = intercept on y-axis
• Determine calibration curve metrics
o PCR efficiency (uses calibration curve slope)
o R^2 (coefficient of determination)
• Determine PCR efficiency: $\%\,\mathbf{E} = \mathbf{100} \times (-\mathbf{1} + \mathbf{10}^{\frac{\mathbf{1}}{\boldsymbol{slope}}})$
• Check that values fall within required range
o R^2 >0.98
o PCR efficiency 100% +/- 15%
</td></tr>
<tr><td>3</td><td>Copy number determination and calculate % horse adulteration</td></tr>
<tr><td>3a</td><td>
$\mathbf{Copy\ number} = \mathbf{10}^{\frac{\boldsymbol{C_q - intercept}}{\boldsymbol{slope}}}$

Test sample:
$\mathrm{H_c} = 10^{\frac{31.13-37.793}{-3.507}} = 79.97$ copies
$\mathrm{M_c} = 10^{\frac{24.33-38.044}{-3.515}} = 7963.01$ copies
</td></tr>
<tr><td></td><td>
• 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
</td></tr>
<tr><td>3b</td><td>
$\%\ \mathbf{Horse\ adulteration\ content} = \frac{\boldsymbol{H_c}}{\boldsymbol{M_c}} \times \mathbf{100}$

Test sample:
$\%\ \mathrm{Horse\ adulteration\ content} = \frac{79.97}{7963.01} \times 100 = 1.00\%$
</td></tr>
<tr><td></td><td>
• Perform relative quantitative analysis to calculate % horse:mammalian DNA ratio
</td></tr>
</table>

11.2. Appendix 2. Example Analytical Decision Tree



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