# Inter-laboratory collaborative trial of real-time PCR method phase 2: Appendix 1, instructions to participants and SOP

(Reproduced exactly as provided originally to the participants, including the sub-Appendix 1. References in this entire Appendix are not numbered.)

Collaborative trial MA0201 practical instructions
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## 1. Introduction

The UK/ EU Horse-meat scandal of 2013 highlighted the lack of traceability in the food supply chain and increased the risk of potentially harmful ingredients within the contaminating meats. As the first step to enforcing tighter measures on the sale of beef products within the UK, Defra commissioned through LGC, the development of a real-time PCR method for the detection and accurate quantification of horse DNA contamination in raw beef (FA0135). This method was successfully validated through an international collaborative trial (Burns M., et al. 2018) and is undergoing standardisation through the CEN Technical Committee on Food Authenticity (CEN TC460).

A requirement for the testing of pork meat in raw and processed beef and horse and pork DNA in raw and processed beef lead to the development of a second real-time PCR method funded by Defra (FA0171). These methods are now required to be validated by a full-scale inter-laboratory collaborative trial.

## 2. Scope

The method described in this SOP is to provide practical instructions on the set-up of the real-time PCR method and quantification of (i) horse DNA in raw beef (ii) horse DNA in processed beef (iii) pork DNA in raw beef and (iv) pork DNA in processed beef.

The purpose of this trial is for the quantification of DNA originating from contaminant meat (horse and/or pork) in raw and processed beef background, relative to mammalian DNA. We have provided 30 blind test samples that consist of a variety of percent contamination DNA in mammalian DNA (% w/w). The samples are labelled PCR_01 to PCR_30 and will give no indication on their respective percent contamination.

The method consists of building a set of calibration curves for the horse specific target and the mammalian (background) specific target using 100% horse DNA, as well as the pork specific target and the mammalian (background) specific target using 100% pork DNA. These calibration curves are based on estimates of the genome equivalent copy number for each horse and pork

and will then be used to accurately quantify the 30 blind test samples. The final result is the quantification of the contaminant DNA relative to the mammalian DNA and is expressed as a ratio of the number of genome equivalents for the contaminant relative to the total number of mammalian genome equivalents in the sample.

# 3. Safety

Laboratory safety procedures must be followed at all times. Only trained and competent staff must undertake this procedure. Ensure all appropriate PPE is worn and the relevant laboratory COSHH and risk assessments are consulted prior to undertaking this method.

# 4. Method performance characteristics

## 4.1. DNA supplied

The standards of 100% horse and 100% pork DNA were extracted from authenticated horse and pork meat respectively, using the same methodology.

The test samples of contaminant (horse or pork) meat in raw and processed beef background were first mixed by weight to achieve a variety of percent contamination (% w/w). The test samples were then extracted using the same methodology as the standards.

The extracted DNA quality and quantity were assessed by a spectrophotometer (Nanodrop 1000) and all DNA passed quality control. A260/A230 ratio ? 1.8 and A260/A280 ratio ? 1.8 and 2.0.

## 4.2. Calibration Curves

This procedure was internally validated at Fera Science Ltd. following the methodology as developed by LGC. The validation was carried out on QuantStudio 6 Flex Real-time PCR system (Applied Biosystems).

Calibration curves for horse and pork are made from 100% horse DNA or pork DNA respectively. The horse and pork calibration curves cover a dynamic range of 40 ng/ µl to 9.8 pg/ µl. The estimated genome equivalent at 40 ng/ µl for both contaminant meats is 31,508 copies based on 1 haploid copy of the horse genome being 2474.9 Mb (Equus caballus NCBI genomes 2018) and the pig genome being 2458.2 Mb (Sus scrofa NCBI genomes 2018).

Within the dynamic range of the calibration curves the slope is between -3.6 and -3.1 and the coefficient of determination (R2) is ?0.98.

## 4.3. Limit of detection

The limit of detection (LOD) is defined as the lowest target concentration which is detectable 95% of the time. The LOD for the horse and pork genome was ?9 pg/ µl (estimated genome equivalent of 7 copies), based on processed horse in beef and pork in beef samples.

## 4.4. Limit of quantification

The limit of quantification (LOQ) is defined as the lowest relative amount of contaminant meat (horse or pork) which is detectable 95% of the time. The LOQ for the horse and pork genome was ?0.1% w/w of processed horse in processed beef and processed pork in processed beef.

# 5. Materials supplied

On receipt of the Collaborative trial MA0201 kit please ensure the integrity of the packaging as well as all the contents.  Any tubes which are damaged, or where samples have leaked must be reported so a replacement sample can be dispatched.  Please report any issues with the kit contents to info@fapas.com, include the exact name on the label of the tube as well as your laboratory identifying information.

### 5.1 DNA samples

30 samples are provided as DNA extracts prepared from contaminant meats in raw and processed beef in varying proportions.

| Sample | Volume | Consumable |
|---|---|---|
| PCR_0 to PCR_30 | 150 µl | x1 Skirted tube (0.5ml) |

### 5.2 Reagents/Consumables

| Reagent | Volume | Consumable |
|---|---|---|
| Horse forward and reverse primers | 150 µl | x2 Skirted tube (0.5ml) |
| Pork forward and reverse primers | 150 µl | x2 Skirted tube (0.5ml) |
| Myostatin forward and reverse primers | 300 µl | x2 Skirted tube (0.5ml) |
| Horse probe | 350 µl | x1 Skirted tube (0.5ml) |
| Pork probe | 350 µl | x1 Skirted tube (0.5ml) |
| Myostatin probe | 650 µl | x1 Skirted tube (1.5ml) |
| Master mix (taq polymerase, dNTPs, buffer) | 9 ml | x2 Skirted tube (10ml) |
| DNA Standards (DNA in buffer) | 350 µl | x2 Skirted tube (0.5ml) |

Primer and probes sequences can be found in Appendix 1.

## 6. Materials required

### 6.1 Equipment

- Standard laboratory personal protective equipment (PPE)
- Disinfectant
- Vortex mixer
- Benchtop centrifuge for centrifuging tubes
- Benchtop centrifuge for centrifuging PCR plates
- Calibrated micropipettes ranging from 1 µl to 1000 µl
- Real-time PCR machine

### 6.2 Consumables

- Optical 96 well clear PCR reaction plates
- Plastic microtubes
- Micropipette tips to fit the required micropipettes

## 7. Procedure

### 7.1 Laboratory organisation

Fera is an ISO17025 accredited laboratory and in accordance with our Quality policy we adhere to a unidirectional workflow plan that minimises cross contamination. The laboratory is organised into discrete spatially separated areas that contains dedicated equipment and PPE. All surfaces and equipment are decontaminated with appropriate disinfection agents and there are established waste disposal procedures. All equipment is regularly calibrated and serviced.

## 7.2 Experimental conditions

1. A suggested 96 well plate plan that includes as singleplex the horse or pork assay and the mammalian assay can be found in Appendix 2. The plan also incorporates samples in triplicate, Standards (horse or pork DNA) and non-template controls (NTC).
2. In order to quantify all test samples, they must be run for both the horse specific assay alongside the mammalian assay and the pork specific assay alongside the mammalian assay. I.e. you must run 3 plates for each assay.
3. In a clean lab briefly vortex all PCR reagent tubes in the kit and pulse spin to collect contents at the bottom of the tube.
4. Make up a working master mix solution for each assay as per Table 1.

**Table 1: Real-time PCR reaction composition.** Reaction composition for each assay (horse, pork and myostatin) for a total reaction volume of 25 µl. Volumes are provided for 1X and for 156X which corresponds to all PCR reactions required per assay. The template DNA volumes for the standards and for the test samples is 5 µl. MGW: Molecular grade water.

| PCR reagent | 1x per assay | 156x per assay |
|---|---|---|
| 2x Universal Master Mix | 12.5 | 1950 |
| Forward primer (20 µM) | 0.375 | 58.5 |
| Reverse primer (20 µM) | 0.375 | 58.5 |
| Probe (5 µM) | 1 | 156 |
| MGW | 5.75 | 897 |
| Template DNA (Standards and test samples) | 5 | 5 |

5. Transfer 20 µl of the PCR reaction master mix solution into the appropriate wells according to the plate plan.
6. In the sample preparation laboratory prepare a dilution series (S1 to S5) for the horse and pork calibration curves.

6.1. The horse DNA standard is in the tube labelled Horse standard 1. This standard is 100% horse DNA at 40 ng/ µl and 350 µl volume. To prepare the 5 point and 8-fold calibration curve for horse refer to Table 2.

6.2. The pork DNA standard is in the tube labelled Pork standard 1. This standard is 100% pork DNA at 40 ng/ µl and 350 µl volume. To prepare the 5 point and 8-fold calibration curve for pork refer to Table 2.

**Table 2: Dilution series for the horse and pork 5 point 8-fold calibration curve.** This serial dilution is based on an initial 100% horse or pork DNA stock at a concentration of 40 ng/ µl at 350 µl. This dilution series will provide sufficient final volumes for each standard level for all required real-time PCR reactions. MGW: Molecular grade water.

| Standard level | DNA (ng/µl) | Genome equivalent (copies/µl) | Volume stock (µl) | Volume MGW (µl) |
|---|---|---|---|---|
| S1 | 40 | 31508 | 350 | 0 |

| Standard level | DNA (ng/µl) | Genome equivalent(copies/µl) | Volume stock (µl) | Volume MGW (µl) |
|---|---|---|---|---|
| S2 | 5 | 3938.5 | 15 (std 1) | 105 |
| S3 | 0.625 | 492.3 | 15 (std 2) | 105 |
| S4 | 0.078 | 61.5 | 15 (std 3) | 105 |
| S5 | 0.009 | 7.69 | 15 (std 4) | 105 |

7. Briefly vortex all standards and test samples and pulse spin to collect contents at the bottom of the tube.
8. Place all the standards and test samples (PCR_01 to PCR_30) in a rack according to the plate plan. Note: do not dilute test samples, they have already been diluted to 10 ng/ µl.
9. Spike the test samples and the calibrant samples into the appropriate wells according to the plate plan. Seal the plate thoroughly with optical adhesive film, vortex the sealed plate for 30 seconds and centrifuge the plate at 1000 g for 1 minute to collect contents to the bottom of the wells.
10. Place the plate into the real-time PCR machine.
11. Designate the wells with the FAM-NFQ detector per assay.
12. Ensure the correct cycling parameters are chosen (see Table 3 for cycling parameter).

**Table 3: PCR thermal cycling conditions**

| Temperature (c) | Time (s) | Repeat cycles |
|---|---|---|
| 50 | 120 | 1 |
| 95 | 600 | 1 |
| 95 | 15 | 45 |
| 60 | 60 | 45 |

## 7.3 Data Analysis

13. View the graph in log mode and check that the threshold settings are adequate (sigmoidal curve and plateau level). If the data requires manual manipulation refer to the instrument manufacturer's recommendations.

14. If any of the water blank replicates give a positive result the plate should be assessed for probable contamination and a decision made on whether to reject and rerun.

15. Export the results into the Microsoft Excel MA0201 Collaborative_Trial_Results 2022 spreadsheet provided, into the appropriate tables for raw data (e.g. raw data from plate 1 should be in tab Raw_data_plate 1).
16. Repeat step 15 for the second contaminant assay relative to the mammalian assay. I.e. you must input the raw data from the horse and pork assays into the results spreadsheet.

17. The percentage of contaminant DNA in mammalian DNA is expressed as a ratio of the number of genome equivalents of the contaminant relative to the total mammalian genome equivalents present in the sample. The results spreadsheet will automatically generate a calibration curve to the contaminant assay and the mammalian (myostatin) assay to the equation for a straight line (y=mx+c) and the coefficient of determination (R^2).

18. For each assay check the R^2 is above 0.98 and PCR efficiency is between 85 and 115%.

19. The spreadsheet will then automatically calculate the relative quantitative value of contaminant DNA compared to total mammalian DNA for each test sample using the following equation.

% contaminant DNA =  (contaminant genome equivalent copy number)/(mammalian genome equivalent copy number) ×100

20. All % contaminant DNA values will be automatically copied in the Collated data tab.

21. Please report these results for each assay through the Fapas secure portal using your personal account as provided by following instructions available at fapas.com/technical documentation.

# 8. References


- Defra project FA0135 (2013) “Real-time PCR approach for quantitation of horse DNA and study into relevance of expression units (DNA/DNA and w/w tissue).”
- Defra project FA0171 (2018) “Validation of Methods to Quantify Horse and Pork Meat Adulteration in Raw and Processed Beef”.
- Burns, M., Nixon, G., Cowen, S., Wilkes, T. International collaborative trial of a real-time PCR approach for the relative quantitation of horse DNA. Food and Nutrition Open Access, 2018, DOI: 10.31021/fnoa.20181113.