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Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR —

Part 6: Horse DNA detection method

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Analyse de biomarqueurs moléculaires — Détection de matériaux d'origine animale dans les denrées alimentaires et les aliments pour animaux par PCR en temps réel —

Partie 6: Méthode de détection de l'ADN de cheval

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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Introduction

Fraudulent adulteration of meat in food and feed threatens both public safety and commerce. Adulteration can affect those adhering to ethnological dietary rules, economic development and social stability. This document provides a real-time polymerase chain reaction (real-time PCR) analytical method for the identification of meat animal species from nucleic acid present in the ingredients of food and feed.

Animal-derived biological materials in food and feed are detected and identified in the laboratory with the following successive (or simultaneous) steps: preparation of the test portion/sample, nucleic acid extraction and purification, PCR amplification and interpretation of results. This document provides guidance for PCR amplification and interpretation of results, specific to horse DNA detection.

The ISO 20224 series consists of technical specifications that describe specific applications. New species DNA detection methods established in the future will be added as independent parts.

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Molecular biomarker analysis — Detection of animal-derived materials in foodstuffs and feedstuffs by real-time PCR —

Part 6: **Horse DNA detection method**

1 Scope

This document specifies a real-time polymerase chain reaction (real-time PCR) method for the qualitative detection of horse-specific DNA derived from food and feed. It requires the extraction of an adequate amount of PCR amplifiable DNA from the relevant matrix and can be applied to the detection of horse material derived from domestic horse (*Equus caballus*), mule (*Equus caballus* ♀ × *Equus asinus* ♂), hinny (*Equus caballus* ♂ × *Equus asinus* ♀) and zebroid (*Equus caballus* × *Equus simplicidens*). The assay also detects the species Przewalski's horse (*Equus przewalskii*) and zebra (*Equus burchellii*).

The target sequence is an *Equus caballus* isolate Twilight breed thoroughbred chromosome 28, EquCab3.0, whole genome shotgun sequence (i.e. GenBank accession number NC_009171.3)[1], which is present as a single copy per haploid genome. The provided PCR assay for this target has an absolute limit of detection of five copies per reaction, with ≥ 95 % replicability at this concentration ($LOD_{95\,\%}$).

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2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16577, *Molecular biomarker analysis — Terms and definitions*

ISO 20813, *Molecular biomarker analysis — Methods of analysis for the detection and identification of animal species in foods and food products (nucleic acid-based methods) — General requirements and definitions*

ISO 21571, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction*

ISO 24276, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

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4 Scientific basis

DNA is extracted from the test portion by applying a suitable method (see ISO 21571:2005, A.1). The DNA analysis consists of two parts:

— verification of the quality and amplifiability of the extracted DNA using a PCR assay specific for eukaryotes (e.g. 18S rRNA gene) or mammals and poultry (e.g. myostatin gene);

— detection of the horse species-specific DNA sequence of the single-copy *Equus caballus* isolate Twilight breed thoroughbred chromosome 28, EquCab3.0, whole genome shotgun sequence. (GenBank accession number NC_009171.3) in a real-time PCR.

NOTE The copy number of the eukaryotic ribosomal 18S RNA (18S rRNA) gene in a cell varies from several hundred to several thousand, while the specific target sequence in the horse genome and myostatin gene in mammals and poultry genome are single copy. The copy number of the specific target sequence in the horse genome was confirmed by bioinformatics analysis at the whole genome scale (see Annex A) and digital PCR for absolute quantification.

5 Reagents and materials

5.1 General

For this document, only chemicals and water of recognized analytical grade, appropriate for molecular biology, shall be used. Unless stated otherwise, solutions should be prepared by dissolving the corresponding reagents in water followed by autoclave sterilization. For all operations in which gloves are used, gloves shall be powder free. The use of aerosol protected pipette tips (protection against cross-contamination) is recommended.

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5.2 PCR reagents

5.2.1 PCR master mix.

PCR master mix contains thermostable DNA polymerase, pH buffer, KCl, $MgCl_2$, uracil-DNA glycosylase (UDG) and the four dNTPS (dATP, dGTP, dUTP, dCTP) as a dilutable concentrate, which is ready to use.

5.2.2 Oligonucleotides.

The quality of the oligonucleotides shall be sufficient for their use as primers and probes. See Table 1.

Table 1 — Oligonucleotides

Name	DNA sequence of the oligonucleotide	Final concentration in PCR
Specific DNA sequence in *Equus caballus* isolate Twilight breed thoroughbred chromosome 28, EquCab3.0, whole genome shotgun sequence (GenBank accession number NC_009171.3)[a]		
Horse-125bp-F	5′-ACTCATCAAACGCCGCTCTC-3′	400 nmol/l
Horse-125bp-R	5′-GCTGTGAAGACCCCGTTGG-3′	400 nmol/l
Horse-125bp-P	5′-[FAM]-CCAGGGCTCGGTGCTTCCAATCGC-[TAMRA][b]-3′	200 nmol/l

[a] PCR product = 41 821 144 - ACTCATCAAA CGCCGCTCTC GAGATCCGTG CACATCGTTC AATGGAAACT TCATTTTAAA AAAGAGAAAA AGGCGATTGG AAGCACCGAG CCCTGGGTAG CGTGTGCCAA CGGGGTCTTC ACAGC - 41 821 268 - NC_009171.3.

[b] FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine.

Horse-125bp-F is base pairs 41 821 144-41 821 163, Horse-125bp-R is base pairs 41 821 250-41 821 268 and Horse-125bp-P is 41 821 216-41 821 239 of NC_009171.3, horse chromosome 28 DNA sequence. Equivalent reporter dyes and/or quencher dyes can be used if they yield the same or better results.

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6 Apparatus

Requirements concerning apparatus and materials shall follow ISO 20813. In addition to the usual laboratory equipment, the following equipment is required.

6.1 Real-time thermocycler instrument.

A device that amplifies DNA in vitro and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting sufficient emitted fluorescent light of the fluorophore used to perform TaqMan format assays.

7 Procedure

7.1 Preparation of the test portion/sample

The test sample used for DNA extraction shall be representative of the laboratory sample and homogeneous, e.g. by grinding or homogenizing the laboratory sample to a fine mixture. Test portion/sample preparation shall follow the general requirements and specific methods described in ISO 21571 and ISO 20813.

7.2 Preparation of DNA extracts

The extraction/purification and quantification of DNA from the test portion shall follow the general requirements and methods provided in ISO 21571. DNA extraction methods described in ISO 21571:2005, Annex A, are recommended.

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7.3 PCR setup

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7.3.1 Reaction mixes

The method is for a total volume of 25 µl per PCR. The reaction setup is given in Table 2. Reagents shall be completely thawed at room temperature. Each reagent shall be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared to contain all components except for the sample DNA. The required total amount of the PCR reagent mixture prepared depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. The number of sample and control replicates shall follow ISO 20813. Set up the PCR tests as follows:

a) mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial;

b) add 5 µl of each sample DNA (20 ng/µl to 200 ng/µl) or positive DNA target control or extraction blank control or water to the respective reaction vials;

c) mix and centrifuge briefly.

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Table 2 — Reaction setup for the amplification

Total reaction volume	25 µl
Sample DNA (20 ng/µl to 200 ng/µl) or controls	5 µl
2 × PCR master mix[a]	12,5 µl
Primer Horse-125bp-F, c = 10 µmol/l and Horse-125bp-R, c = 10 µmol/l	1,0 µl for each
Probe Horse-125bp-P, c = 10 µmol/l	0,5 µl
Water	to 25 µl

[a] In the collaborative trial, a ready-to-use optimized 2 × PCR master mix containing all of the components, excluding the template and primers, was used. The 2 × PCR master mix contains thermostable DNA polymerase, a blend of dNTPs with dUTP and uracil-UDG to minimize carry-over PCR contamination, and a passive internal reference based on ROX dye. Equivalent products can be used if they yield the same or better results. If necessary, the amounts of the reagents and the temperature-time programme can be adapted.

7.3.2 PCR controls

7.3.2.1 General

PCR controls shall be as described in ISO 24276 and ISO 20813.

7.3.2.2 Inhibition control (reference gene assay)

A reference control gene (e.g. 18S rRNA gene for eukaryotes, myostatin gene for mammals and poultry) PCR assay using sample DNAs shall be performed to test nucleic acid amplifiability and provide control to exclude false-negative results.

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7.3.3 Real-time PCR thermocycler plate set-up

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Transfer the setup reaction vials to the thermocycler. The vials should be arranged to avoid any possible edge temperature variations associated with a particular real-time thermocycler instrument. Start the temperature-time programme.

7.4 Temperature-time programme

The temperature-time programme as outlined in Table 3 was used in the validation study. The use of different reaction conditions and real-time PCR cycles shall be verified. The time for initial denaturation depends on the master mix used.

Table 3 — Temperature-time programme

Step	Parameter		Temperature	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95 °C	10 min	no	1
2	Amplification	Denaturation	95 °C	15 s	no	45
		Annealing and elongation	60 °C	60 s	yes	

8 Accept/reject criteria

8.1 General

A corresponding real-time PCR-instrument-specific data analysis programme shall be used for the identification of PCR products. The amplification results can be expressed differently, depending on the instrument used. In the absence of detectable PCR products (e.g. negative controls), the result shall be expressed as "undetermined", "no amplification" or the maximum number of reaction cycles

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performed. If amplification of the DNA target sequence in a sample (e.g. positive controls) occurred, a sigmoid-shaped amplification curve shall be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold shall be calculated [cycle threshold (C_t) or cycle quantification (C_q)].

If, due to atypical fluorescence measurement data, the automatic interpretation does not provide a meaningful result, it may be necessary to set the baseline and the threshold manually prior to interpreting the data. In such a case, the device-specific instructions provided with the interpretation software shall be followed.

8.2 Identification

The target sequence is considered as detected if:

— horse-specific primers Horse-125bp-F and Horse-125bp-R and the probe Horse-125bp-P produce a sigmoid-shaped amplification curve and a C_t value or C_q value can be calculated;

— PCR control reactions with no added DNA (PCR reagent control, extraction blank control) produce no amplification;

— the amplification controls (positive DNA target control, PCR inhibition control) produce the expected amplification and C_t values (or C_q values).

Trace detections are defined as PCRs with C_t values later than that defined at the target $LOD_{95\ \%}$. In the event of a trace detection or contradictory positive/negative results from different extracts of the same sample, then the sample shall be retested. At least two new extracts shall be prepared from the homogenized laboratory sample. A minimum of 20 PCR replicates shall be conducted across the new extracts (e.g. ten PCR repeats for two extracted DNA, seven PCR repeats for three extracted DNA). The target sequence shall be considered as “detected” if ≥ 95 % of the new extract PCR results show a positive detection. The target sequence shall be considered as “not detected” if < 95 % of the new extract PCR results show a positive detection.

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9 Validation status and performance criteria

9.1 General

Validation followed a two-part process:

a) in-house validation;

b) collaborative trial validation.

9.2 Robustness

The robustness of the method was confirmed for the collaborative trial by changing the reaction conditions for the following factors:

a) real-time PCR instruments (e.g. ABI 7500, BioRad CFX96, ABI 7900 HT Fast, Eppendorf Realplex 4[1)]);

b) reaction volume: 19 µl or 21 µl PCR reagent mixture plus 5 µl sample DNA (20 ng/µl to 200 ng/µl);

c) annealing temperature: 59 °C and 61 °C;

d) primer or probe concentration: both reduced by 30 %.

For each factor tested, the PCRs were analysed in triplicate, each with 20 copies of the target sequence and with 100 copies of the non-target sequence as negative controls. Method performance

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