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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**

*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*

**PROOF / ÉPREUVE**

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CP 401 • Ch. de Blandonnet 8  
CH-1214 Vernier, Geneva  
Phone: +41 22 749 01 11  
Email: [copyright@iso.org](mailto:copyright@iso.org)  
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## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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](http://www.iso.org/directives)).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see [www.iso.org/patents](http://www.iso.org/patents)).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

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

A list of all parts in the ISO 20224 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

## 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 (e.g. GenBank accession number NC\_009171.3)<sup>[1]</sup>, 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<sub>95</sub> %).

### 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 and the following 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/>

## 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 horse genome and myostatin gene in mammals and poultry genome are single copy. The specific target sequence in 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.

### 5.2 PCR reagents

#### 5.2.1 PCR master mix.

PCR master mix contains thermostable DNA polymerase, pH buffer, KCl, MgCl<sub>2</sub>, 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 <i>Equus caballus</i> isolate Twilight breed thoroughbred chromosome 28, EquCab3.0, whole genome shotgun sequence (GenBank accession number NC_009171.3) <sup>a</sup>		
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] <sup>b</sup> -3'	200 nmol/l
<sup>a</sup> PCR product = 41821144 - ACTCATCAAAA CGCCGCTCTC GAGATCCGTG CACATCGTTC AATGGAAACT TCATTTTAAA AAAGAGAAAA AGGCGATTGG AAGCACCGAG CCCTGGGTAG CGTGTGCCAA CGGGGTCTTC ACAGC - 41821268 - NC_009171.3.		
<sup>b</sup> FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine.		

Horse-125bp-F is base pairs 41821144-41821163, Horse-125bp-R is base pairs 41821250-41821268 and Horse-125bp-P is 41821216-41821239 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.



## 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 the emitted fluorescent light of the used fluorophore sufficient 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.

### 7.3 PCR setup

#### 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.

**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 <sup>a</sup>	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

<sup>a</sup> 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.

### 7.3.3 Real-time PCR thermocycler plate set-up

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