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

Part 11: Pigeon DNA detection method

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National foreword

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

Part 11: Pigeon 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 11: Méthode de détection de l'ADN de pigeon

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Foreword

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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 rock pigeon (*Columba livia*) 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 11: Pigeon DNA detection method

1 Scope

This document specifies a real-time polymerase chain reaction (real-time PCR) method for the qualitative detection of pigeon-specific DNA derived from food and feed. The method can be applied to distinguish rock pigeon (*Columba livia*) from other domestic poultry meats (e. g. goose, duck, quail, pheasant). It requires the extraction of an adequate amount of PCR amplifiable DNA from the relevant matrix.

The target sequence is a partial fragment of *Columba livia* breed Danish Tumbler unplaced genomic scaffold, Cliv_1.0 scaffold114 (i.e. GenBank accession number NW_004973337.1),[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 % confidence at this concentration ($LOD_{95\ \%}$).

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 — Vocabulary for molecular biomarker analytical methods in agriculture and food production*

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 terminology databases for use in standardization at the following addresses:

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

— IEC Electropedia: available at https://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 (i.e. 18S rRNA gene) or mammals and poultry (i.e. myostatin gene);

— detection of the pigeon species-specific DNA sequence of the single-copy *Columba livia* unplaced genomic scaffold sequence (i.e. GenBank accession number NW_004973337.1) 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 pigeon genome and myostatin gene in mammals and poultry genome are single copy. The copy number of the specific target sequence in the pigeon 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 reagents 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.

In general, real-time PCR master mix contains thermostable DNA polymerase, dNTPs, $MgCl_2$, KCl, and buffer as a dilutable concentrated mixture, that 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 sequence in *Columba livia* unplaced genomic scaffold sequence (i.e. GenBank accession number NW_004973337.1)[a]		
Pigeon-113bp-F	5′- GCAGTTGTTTAGTCCTCCTGTAAC -3′	400 nmol/l
Pigeon-113bp-R	5′- GGGCTAACATCAAGACGCAAAG -3′	400 nmol/l
Pigeon-113bp-P	5′- [FAM]- CGGACTCCTAAGAGCACTTCTCAGCCTGG -[TAMRA][b]-3′	200 nmol/l

[a] PCR product = 3 716 744 - GCAGTTGTTT AGTCCTCCTG TAACACGGAC TCCTAAGAGC ACTTCTCAGC CTGGCTTTGT TTTCGTCACA CTGTGTATCT GAACCGCCGT TCTTTGCGTC TTGATGTTAG CCC – 3 716 856 - NW_004973337.1

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

Pigeon-113bp-F is base pairs 3 716 744 – 3 716 767, Pigeon-113bp-R is base pairs 3 716 835 – 3 716 856 and Pigeon-113bp-P is 3 716 769 – 3 716 797 of NW_004973337.1, pigeon unplaced genomic scaffold 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 that are 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.

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 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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