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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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Contents

Page

Foreword iv
Introduction v
1 Scope 1
2 Normative references 1
3 Terms and definitions 1
4 Scientific basis 2
5 Reagents and materials 2
5.1 General 2
5.2 PCR reagents 2
5.2.1 PCR master mix. 2
5.2.2 Oligonucleotides. 2
6 Apparatus 3
6.1 Real-time thermocycler instrument. 3
7 Procedure 3
7.1 Preparation of the test portion/sample 3
7.2 Preparation of DNA extracts 3
7.3 PCR setup 3
7.3.1 Reaction mixes 3
7.3.2 PCR controls 4
7.3.3 Real-time PCR thermocycler plate set-up 4
7.4 Temperature-time programme 4
8 Accept/reject criteria 4
8.1 General 4
8.2 Identification 5
9 Validation status and performance criteria 5
9.1 General 5
9.2 Robustness 5
9.3 Reproducibility 6
9.4 Sensitivity 6
9.5 Specificity 9
10 Test report 11
Annex A (informative) **BlastN +2.12.0 results for query of GenBank RefSeq genome (refseq_genomes) and whole-genome shotgun contigs (wgs)** 12
Annex B (informative) **Members of the *Columbidae* family and its family tree established with available public genomic sequences** 14
Bibliography 24

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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 document 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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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.

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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 ($LOD_{95\,\%}$).

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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.

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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.

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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.

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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 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

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.

Table 2 — Reaction setup for the amplification

Total reaction volume	25 µl
Sample DNA[a] or controls	5 µl
2 × PCR master mix[b]	12,5 µl

[a] The amount of DNA in one reaction can be up to 200 ng, but the recommendation amount is less than 200 ng per reaction.

[b] 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.

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Table 2 *(continued)*

Primer Pigeon-113bp-F, c = 10 µmol/l and Pigeon-113bp-R, c = 10 µmol/l	1,0 µl for each
Probe Pigeon-113bp-P, c = 10 µmol/l	0,5 µl
Water	to 25 µl

[a] The amount of DNA in one reaction can be up to 200 ng, but the recommendation amount is less than 200 ng per reaction.

[b] 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 (i.e. 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

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

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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	UNG activation[a]		50 °C	2 min	no	1
2	Initial denaturation		95 °C	10 min	no	1
3	Amplification	Denaturation	95 °C	15 s	no	45
		Annealing and elongation	60 °C	60 s	yes	

a UNG (Uracil-N-Glycosylase) activation is mandatory if UDG-glycosylase is included in mastermix and optional if UDG-glycosylase is not included in mastermix.

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

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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 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:

— pigeon-specific primers Pigeon-113bp-F and Pigeon-113bp-R and the probe Pigeon-113bp-P, produce a sigmoid-shaped amplification curve and a C_t value or C_q value ≤$LOD_{95\ \%}$ 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;

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

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

1) These are examples of a suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

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