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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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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.*

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.

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.

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/

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.

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.

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
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.

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	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 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.

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]);

1) These are examples of 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.

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 %.

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 remained satisfactory for both samples and negative controls under the changed conditions for each changed factor.

9.3 Reproducibility

The reproducibility of the method was verified in a collaborative trial with 12 participants, organized by the Technical Center for Animal, Plant and Food Inspection and Quarantine, Shanghai Customs in accordance with the IUPAC protocol[2] and the BVL guidelines.[3] Participants received 12 DNA samples for the evaluation of false-positive and false-negative rates. All samples were labelled with randomized coding numbers and consisted of six replicate samples. The 12 DNA samples were:

— six vials of pigeon DNA solution, 10 copies/µl;

— six vials of bovine DNA solution, 20 copies/µl.

The copy numbers were determined using the real-time PCR of this method and serial dilutions of plasmid DNA containing the target sequence. The concentration of the plasmid-DNA (copies/µl) was measured by digital PCR.

Participants received a PCR master mix and the oligonucleotides (primers and probes) from the collaborative test organizer to conduct the PCR experiments.

Pigeon and bovine genomic DNA were extracted from pigeon meat and bovine meat, respectively, and then adjusted with 0,2 × TE buffer to a nominal concentration of 10 copies/µl for pigeon DNA and 20 copies/µl for bovine DNA, respectively.

The collaborative trial was designed to determine false-positive and false-negative rates. Each DNA sample was tested by the participants in a single PCR test with 5 µl of the respective DNA solution, using the procedure and the conditions given in Tables 2 and 3. The results of the collaborative trial are listed in Table 4.

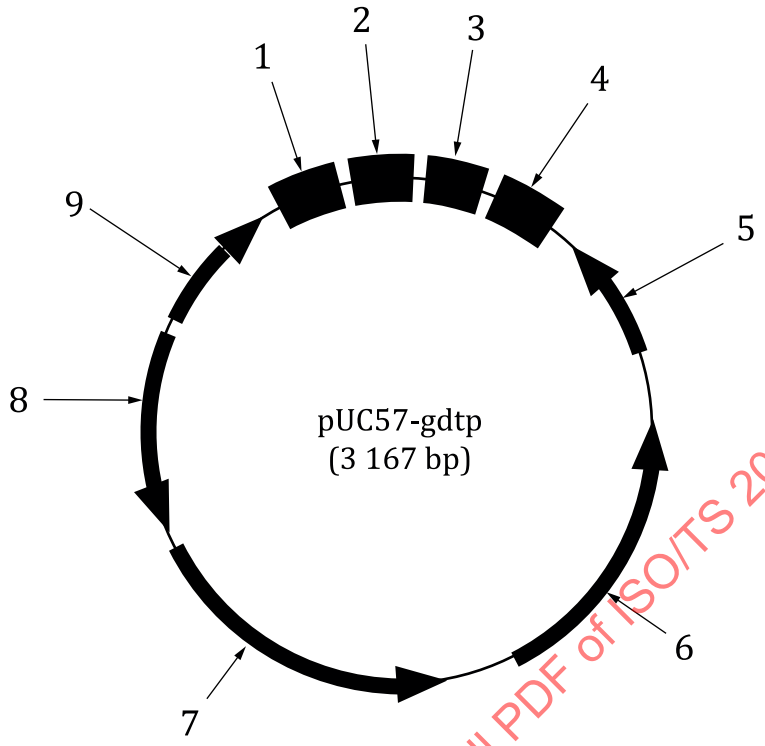
Table 4 — Results of the collaborative trial

Number of laboratories	12
Number of laboratories submitting results	12
Number of samples per laboratory	12
Number of accepted results	144
Number of accepted samples containing pigeon material	72
Number of accepted samples not containing pigeon material	72
False-positive results	0
False-positive results (in %)	0
False-negative results	0
False-negative results (in %)	0

9.4 Sensitivity

The absolute limit of detection ($LOD_{95\ \%}$) for the method is five DNA copies. The collaborative trial of the pigeon detection method was carried out at the same time as collaborative trials for the goose, turkey, and duck detection methods. Goose (*Anser anser* domesticus), duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*) and rock pigeon (*Columba livia*) target DNA sequences were synthesized and cloned into the pUC57 vector (2 710 bp in length, GenBank/EMBL accession number Y14837). This constructed plasmid

pUC57-gdtp (3 167 bp in length) was sequenced to ensure that only one copy of the goose, duck, turkey and rock pigeon target DNA sequence was inserted (see Figure 1). No deletion or insertion mutations were found in the inserted sequences (see Figure 2). The target sequences of corresponding PCR methods are indicated.



Key

1 nt 1-121 = goose amplicon (121 bp)
2 nt 122~226 = duck amplicon (105 bp)
3 nt 227~344 = turkey amplicon (118 bp)
4 nt 345~457 = pigeon amplicon (113 bp)
5 M13 reverse promoter
6 ColE1 origin of replication
7 β-lactamase gene (ampicillin resistance gene)
8 ampicillin resistance gene promoter
9 M13 forward promoter

Figure 1 — Map of the multi-target DNA plasmid

1	ACGAGGATAG	GTTGTGACAG	CTGACTCTGT	TCAGCCTTGC	GAAGACCTTA
51	TGCTGTCTAC	AATTACCTAA	TTGGAGGATA	TAGAATTATA	GAATCATATA
101	GAGAAGACGA	CACAGAGATT	CTCTTCACAA	GCAGGGTCTA	ATGGAAGACT
151	TGCTGGCCTG	CTCTCTACTG	GTGATGATGT	GGTGAGATGC	GTGCTGTGCC
201	TCTCTTCCTC	TGGACCTTCT	GCCAAGTGAA	CAAATCCACT	TCCCTTTAAC
251	CTCAGGAACA	TCCAGCATAT	TGGTAAACAG	CGGGATGTGG	GGGTGTGGCT
301	GCGGCTCGTC	ATCACCTGCA	GCTCACTTGT	GCAGCAGCTA	ATGAGCAGTT
351	GTTTAGTCCT	CCTGTAACAC	GGACTCCTAA	GAGCACTTCT	CAGCCTGGCT
401	TTGTTTTCGT	CACACTGTGT	ATCTGAACCG	CCGTTCTTTG	CGTCTTGATG
451	TTAGCCC				

Key

single bold underline	nt 1~121 = goose amplicon (121 bp)
double underline	nt 122~226 = duck amplicon (105 bp)
dashed underline	nt 227~344 = turkey amplicon (118 bp)
dotted underline	nt 345~457 = pigeon amplicon (113 bp)

Figure 2 — Complete sequence of nucleotides (nt) and annotation of the insertion in plasmid pUC57

Each participant in the collaborative trial received a solution containing plasmid pUC57 DNA adjusted to 1 000 copies/µl of the target sequence (see Figures 1 and 2) in 20 ng/µl sonicated salmon sperm DNA. The concentration was measured before distribution by digital PCR (QX100 Droplet Digital PCR System[2)]). Serial dilutions were produced by the 12 laboratories in the range of 0,02 copies/µl to 4 copies/µl using 0,2 × TE buffer containing 20 ng/µl sonicated salmon sperm DNA. Each participant measured six replicates per concentration level. A positive result was achieved for five copies per PCR in 72 out of 72 tests (see Table 5).

Probability of detection (POD) describes the probability that PCR amplification will take place at a given number of copies of the target sequences (see ISO/TS 16393[4]). Qualitative data generated across all laboratories and dilution levels (see Table 5) was used to determine the POD = 0,95 of the detection method (see Table 6) as described in Reference [3]. Standard deviation was determined to be 0,29 and the $LOD_{95\ \%}$ was 3,1 copies; both parameters well below the required maximum of 1 and 20 copies, respectively[5].

Table 5 — Collaborative trial results for the limit of detection ($LOD_{95\ \%}$)

Copy number of specific DNA sequence in pigeon genomic sequence per PCR (nominal)	Number of positive results (C_t < 45) out of 72 results
20	72
10	72
5	72
2	66
1	55
0,5	33
0,1	17

2) This is a product supplied by Bio-Rad GmbH. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Table 6 — Collaborative trial results for the probability of detection (POD)

Parameter		Specific DNA sequence in pigeon genomic sequence
Number of laboratories		12
Number of PCR replicates per dilution level		6
POD curve	The mean probability of detection across laboratories (LPOD)	0,82
	95 % confidence interval for LPOD	0,77 to 0,86
	Slope b relative to the ideal POD curve (b = 1)	1,13
	Laboratory standard deviation, σ_L	0,29
$LOD_{95\,\%}$ (in copies)	Theoretical median laboratory	3,1

9.5 Specificity

A representative sequence from *Columba livia* unplaced genomic scaffold sequence (i.e. GenBank accession number NW_004973337.1) was selected as a PCR target according to the alignment results among reference genomes of various bird species.[1] Primers and probes were designed and optimized using primer-probe selection and optimization software.

The theoretical exclusive specificity of *Columba livia* unplaced genomic scaffold sequence's primers and probes was analysed for homology to other species using the BLASTN program.[6] The 113-bp sequence used as query is part of the NCBI accession number NW_004973337.1 (nucleotides position: 3 716 744 – 3 716 856). Similarity search results are given in Annex A. There was no homology with other genes and genera.

The assays specified in Table 7 were established with DNA from different species (about 200 ng/PCR). Theoretically expected data were established by queries in public NCBI databases[6].

The inclusive specificity was tested against eight breeds of *Columba livia* Carneau (Belgium and France, meat), Dewlap (Middle East, homing) Egyptian swift (Egypt, show and flying), Feral (Europe, meat), Ice (Germany, show), Texan (France, egg), Silver King (USA, meat) and White King (USA, meat). At approximate 100 copies of target DNA, all of the breed samples were detected with the expected positive signals and amplification curves. Inclusivity of the 113 base target sequence was also evaluated using the BLASTN program against the GenBank whole animal genomes database. Results indicating that the 113 base target sequence is unique for pigeon are provided in Annex A.

The members of the *Columbidae* family[7] and its family tree established with available public genomic sequences are shown in Figure B.1. The rock pigeon (*Columba livia*) is the ancestor of domesticated pigeons. Due to the limited genomic sequence data available in this family, there are uncertainty of possible cross-detection between the wild and domesticated representation.

Table 7 — Specificity of the target pigeon genomic sequence detection method

Species test		Theoretically expected	Experimental confirmation
Animal	Bison (*Bison bison*)	N	N
	Camel (*Camelus bactrianus*)	N	N
	Carp (*Cyprinus carpio*)	N	N
	Cat (*Felis catus*)	N	N
	Cattle (*Bos taurus*)	N	N
	Chicken (*Gallus gallus*)	N	N
	Domestic goose (*Anser anser* domesticus)	N	N
	Domestic turkey (*Meleagris gallopavo*)	N	N
	Dog (*Canis familiaris*)	N	N
	Donkey (*Equus asinus*)	N	N
	Elk (*Cervus canadensis*)	N	N
	Goat (*Capra hircus*)	N	N
	Goldfish (*Carassius auratus*)	N	N
	Horse (*Equus caballus*)	N	N
	Indian zebu (*Bos indicus*)	N	N
	Japanese wood pigeon (*Columba janthina*)	N	N[a]
	Mallard Duck (*Anas platyrhynchos*)	N	N
	Mouse (*Mus musculus*)	N	N
	Muscovy duck (*Cairina moschata*)	N	N
	Ostrich (*Struthio camelus*)	N	N
	Pheasant (*Phasianus colchicus*)	N	N
	Pig (*Sus scrofa* domesticus)	N	N
	Quail (*Coturnix coturnix*)	N	N
	European rabbit (*Oryctolagus cuniculus*)	N	N
	Rainbow trout (*Oncorhynchus mykiss*)	N	N
	Rat (*Rattus norvegicus*)	N	N
	Rhesus macaque (*Macaca mulatta*)	N	N
	Rock pigeon (*Columba livia*)	Pos	Pos
	Sheep (*Ovis aries*)	N	N
	Eastern spot-billed duck (*Anas zonorhyncha*)	N	N
	Swan goose (*Anser cygnoides* domesticus)	N	N
	Eastern spotted dove (*Streptopelia chinensis*)	N	N
	Water buffalo (*Bubalus bubalis*)	N	N
	Wild turkey (*Meleagris Ocellata*)	N	N
	Yak (*Bos mutus*)	N	N
Human	Human (*Homo sapiens*)	N	N

Key

Pos: positive; N: negative

NOTE [a] In place of a biological sample of Japanese wood pigeon (*Columba janthina*) for experimental testing, the high-homology 113 base target sequence of accession number BMBC01005847.1 (7 810 bp – 7 922 bp) identified by BLASTN was synthesized and cloned in plasmid pUC57.

Table 7 *(continued)*

Species test		Theoretically expected	Experimental confirmation
Plant	Alfalfa (*Medicago sativa*)	N	N
	Corn (*Zea mays*)	N	N
	Rapeseed (*Brassica napus*)	N	N
	Rice (*Oryza sativa*)	N	N
	Sorghum (*Sorghum bicolor*)	N	N
	Soya (*Glycine max*)	N	N
	Wheat (*Triticum aestivum*)	N	N

Key

Pos: positive; N: negative

NOTE [a] In place of a biological sample of Japanese wood pigeon (*Columba janthina*) for experimental testing, the high-homology 113 base target sequence of accession number BMBC01005847.1 (7 810 bp – 7 922 bp) identified by BLASTN was synthesized and cloned in plasmid pUC57.

10 Test report

The test report should be prepared as specified in ISO 20813 and other applicable standards (e.g. ISO 24276).

Annex A
(informative)

BlastN +2.12.0 results for query of GenBank RefSeq genome (refseq_genomes) and whole-genome shotgun contigs (wgs)

A.1 Query

A.1.1 Query ID: NW_004973337.1 (bp 3 716 744 – 3 716 856).

A.1.2 Description: *Columba livia* breed Danish Tumbler unplaced genomic scaffold, Cliv_1.0 scaffold114.

A.1.3 Molecule type: nucleic acid.

A.1.4 Query length: 113.

A.2 Descriptions

See Table A.1.

Table A.1 — Descriptions

Description	Max score	Total score	Query cover%	E value	% Ident	Accession	Accession length	Standard databases	Organism
Columba livia breed Danish Tumbler unplaced genomic scaffold, Cliv_1.0 scaffold114	209	209	100	6e-51	100	NW_004973337.1	4234233	GenBank RefSeq Genome (refseq_genomes)	birds (taxid: 8782)
Columba janthina nitens DNA, Cojn_5847, whole genome shotgun sequence	176	176	100	2e-42	94,69	BMBC01005847.1	15662	Whole-genome Shotgun Contigs (wgs)	birds (taxid: 8782)

A.3 Alignments

Columba livia breed Danish Tumbler unplaced genomic scaffold, Cliv_1.0 scaffold114 .

Sequence ID: NW_004973337.1

Length: 4 234 233 Number of matches: 1 Range 1: 3 716 744 to 3 716 856

Score	Expect	Identities	Gaps	Strand
209 bits(113)	6e-51()	113/113(100 %)	0/113(0 %)	Plus/Plus

```
Query   1         GCAGTTGTTTAGTCCTCCTGTAACACGGACTCCTAAGAGCACTTCTCAGCCTGGCTTTGT   60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   3716744   GCAGTTGTTTAGTCCTCCTGTAACACGGACTCCTAAGAGCACTTCTCAGCCTGGCTTTGT   3716803

Query   61        TTTCGTCACACTGTGTATCTGAACCGCCGTTCTTTGCGTCTTGATGTTAGCCC   113
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   3716804   TTTCGTCACACTGTGTATCTGAACCGCCGTTCTTTGCGTCTTGATGTTAGCCC   3716856
```

Columba janthina nitens DNA, Cojn_5847, whole genome shotgun sequence.

Sequence ID: BMBC01005847.1

Length: 15 662 Number of matches: 1 Range 1: 7 810 to 7 922

Score	Expect	Identities	Gaps	Strand
176 bits(95)	2e-42	107/113(95 %)	0/113(0 %)	Plus/Plus

```
Query   1         GCAGTTGTTTAGTCCTCCTGTAACACGGACTCCTAAGAGCACTTCTCAGCCTGGCTTTGT   60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   7922      GCAGTTGTTTAGTCCTCCTGTAACACGGACTGTTAAGAGCACTTCTCAGTCTGGCTTTGT   7863

Query   61        TTTCGTCACACTGTGTATCTGAACCGCCGTTCTTTGCGTCTTGATGTTAGCCC   113
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   7862      TTTCGTCACACTGTGTATCTGAACTGCCGTTCTTTGCGTCTTGGTGTTATCCC   7810
```

Annex B
(informative)

Members of the *Columbidae* family and its family tree established with available public genomic sequences

B.1 Members of the *Columbidae* family

Table B.1 — Member of the *Columbidae* family

Genus No.	Genus	Species	Accession number of available public genomic sequence
1	*Alectroenas*	*madagascariensis*	
		nitidissimus	
		pulcherrimus	
		sganzini	
2	*Caloenas*	*maculata*	
		nicobarica	VZSB00000000.1 (wgs)
3	*Chalcophaps*	*indica*	
		longirostris	
		stephani	
4	*Claravis*	*pretiosa*	
5	*Columba*	*albinucha*	
		albitorques	
		argentina	
		arquatrix	
		bollii	
		delegorguei	
		elphinstonii	
		eversmanni	
		guinea	
		hodgsonii	
		iriditorques	
		janthina	BMBC01005847.1 (wgs)
		jouyi	
		junoniae	
		larvata	
		leucomela	
		leuconota	
		livia	NW_004973337.1 (genome)
		malherbii	
		oenas	
		oliviae	
		pallidiceps	

Table B.1 *(continued)*

Genus No.	Genus	Species	Accession number of available public genomic sequence
		palumboides	
		palumbus	
		pollenii	
		pulchricollis	
		punicea	
		rupestris	
		sjostedti	
		thomensis	
		torringtoniae	
		trocaz	
		unicincta	
		versicolor	
		vitiensis	
6	*Columbina*	*buckleyi*	
		cruziana	
		cyanopis	
		inca	
		minuta	
		passerina	
		picui	VYZG00000000.1 (wgs)
		squammata	
		talpacoti	
7	*Cryptophaps*	*poecilorrhoa*	
8	*Didunculus*	*strigirostris*	
9	*Drepanoptila*	*holosericea*	
		aenea	
		aurorae	
		badia	
		bakeri	
		basilica	
		bicolor	
		brenchleyi	
		carola	
		chalconota	
		cineracea	
		concinna	
		cuprea	
		finschii	
		forsteni	
		galeata	
		geelvinkiana	
		goliath	
		lacernulata	

Table B.1 *(continued)*

Genus No.	Genus	Species	Accession number of available public genomic sequence
10	*Ducula*	*latrans*	
		luctuosa	
		melanochroa	
		mindorensis	
		mullerii	
		myristicivora	
		neglecta	
		oceanica	
		oenothorax	
		pacifica	
		perspicillata	
		pickeringii	
		pinon	
		pistrinaria	
		poliocephala	
		radiata	
		rosacea	
		rubricera	
		rufigaster	
		spilorrhoa	
		subflavescens	
		whartoni	
		zoeae	
11	*Ectopistes*	*migratorius*	
12	*Gallicolumba*	*criniger*a	
		keayi	
		luzonica	
		menagei	
		platenae	
		rufigula	
		tristigmata	
13	*Geopelia*	*cuneata*	
		humeralis	
		maugeus	
		placida	
		striata	
14	*Geophaps*	*plumifera*	
		scripta	
		smithii	
		caniceps	
		chrysia	
		leucometopia	
		montana	

Table B.1 *(continued)*

Genus No.	Genus	Species	Accession number of available public genomic sequence
15	*Geotrygon*	*mystacea*	
		purpurata	
		saphirina	
		versicolor	
		violacea	
16	*Goura*	*cristata*	
		scheepmakeri	
		sclaterii	
		victoria	
17	*Gymnophaps*	*albertisii*	
		mada	
		stalkeri	
18	*Hemiphaga*	*chathamensis*	
		novaeseelandiae	
19	*Henicophaps*	*albifrons*	
		foersteri	
20	*Leptotila*	*battyi*	
		cassinii	
		conoveri	
		jamaicensis	
		megalura	
		ochraceiventris	
		pallida	
		plumbeiceps	
		rufaxilla	
		verreauxi	
		wellsi	
21	*Leptotrygon*	*veraguensis*	
22	*Leucosarcia*	*melanoleuca*	
23	*Lopholaimus*	*antarcticus*	

Table B.1 *(continued)*

Genus No.	Genus	Species	Accession number of available public genomic sequence
24	*Macropygia*	*amboinensis*	
		cinnamomea	
		doreya	
		emiliana	
		macassariensis	
		mackinlayi	
		magna	
		modiglianii	
		phasianella	
		ruficeps	
		rufipennis	
		tenuirostris	
		timorlaoensis	
		unchall	
25	*Metriopelia*	*aymara*	
		ceciliae	
		melanoptera	
		morenoi	
26	*Microgoura*	*meeki*	
27	*Nesoenas*	*mayeri*	
		picturatus	
		rodericanus	
28	*Ocyphaps*	*lophotes*	
29	*Oena*	*capensis*	
30	*Otidiphaps*	*nobilis*	
31	*Pampusana*	*beccarii*	
		canifrons	
		erythroptera	
		ferruginea	
		hoedtii	
		jobiensis	
		kubaryi	
		norfolkensis	
		rubescens	
		salamonis	
		sanctaecrucis	
		stairi	
		xanthonura	
32	*Paraclaravis*	*geoffroyi*	
		mondetoura	

Table B.1 *(continued)*

Genus No.	Genus	Species	Accession number of available public genomic sequence
33	*Patagioenas*	*araucana*	
		caribaea	
		cayennensis	
		corensis	
		fasciata	LSYS00000000.1 (wgs)
		flavirostris	
		goodsoni	
		inornata	
		leucocephala	
		maculosa	
		nigrirostris	
		oenops	
		picazuro	
		plumbea	
		speciosa	
		squamosa	
		subvinacea	
34	*Petrophassa*	*albipennis*	
35	*Pezophaps*	*solitaria*	
36	*Phapitreron*	*amethystinus*	
		brunneiceps	
		cinereiceps	
		leucotis	
37	*Phaps*	*chalcoptera*	
		elegans	
		histrionica	
		alligator	
		arcanus	
		aurantiifrons	
		bernsteinii	
		chalcurus	
		chrysogaster	
		cinctus	
		coralensis	
		coronulatus	
		dohertyi	
		dupetithouarsii	
		eugeniae	
		fischeri	
		granulifrons	
		greyi	
		gularis	
		hernsheimi	