TECHNICAL SPECIFICATION

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

Part 9: Goose 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 9: Méthode de détection de l'ADN d'oie

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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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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.

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 to detect domestic commercial breeds of swan goose (*Anser cygnoides* domesticus) and domestic goose (*Anser anser* domesticus) and interpretation of results. Cross detection of *Anser brachyrhynchus*, *Anser indicus*, *Branta canadensis*, *Cygnus atratus*, *Cygnus buccinator*, *Cygnus cygnus*, *Cygnus olor*, *Nettapus auritus*, *Oxyura jamaicensis* and *Stictonetta naevosa* of Anseriformes is expected. The method can be applied to distinguish domestic goose from domestic chicken, duck and turkey which are most common adulterants of foie gras.[1] The method is also able to differentiate domestic goose from other high-end domestic poultry meats (quail, pigeon, pheasant).

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 9: Goose DNA detection method

1 Scope

This document specifies a real-time polymerase chain reaction (real-time PCR) method for the qualitative detection of goose-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 goose material derived from domestic breeds of swan goose (*Anser cygnoides* domesticus) and domestic goose (*Anser anser domesticus*). Cross detection of *Anser brachyrhynchus*, *Anser indicus*, *Branta canadensis*, *Cygnus atratus*, *Cygnus buccinator*, *Cygnus cygnus*, *Cygnus olor*, *Nettapus auritus*, *Oxyura jamaicensis* and *Stictonetta naevosa* of Anseriformes is expected.

The method can be applied to distinguish domestic breeds of swan goose (*Anser cygnoides domesticus*) and domestic goose (*Anser anser domesticus*) from domestic chicken, duck and turkey which are the most common adulterants of foie gras.[1] It is also able to differentiate the domestic goose from other high-end domestic poultry meats (quail, pigeon, pheasant).

The target sequence is a partial fragment of the *Anser cygnoides* isolate SCWG-2014 breed Sichuan white goose unplaced genomic scaffold, GooseV1.0 scaffold320 (i.e. GenBank accession number NW_025927981.1)[2], 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 goose species-specific DNA sequence of the single-copy *Anser cygnoides* isolate SCWG-2014 breed Sichuan white goose unplaced genomic scaffold, GooseV1.0 scaffold320 (i.e. GenBank accession number NW_025927981.1) 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 goose genome and myostatin gene in mammals and poultry genome are single copy. The copy number of the specific target sequence in *Anser cygnoides* 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 concentration, which is ready to use.

Commercial real-time PCR master mix may be used.

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 *Anser cygnoides* isolate SCWG-2014 breed Sichuan white goose unplaced genomic scaffold (GenBank accession number NW_025927981.1)[a]		
Goose-121bp-F	5′-ACGAGGATAGGTTGTGACAGC-3′	400 nmol/l
Goose-121bp-R	5′-GAATCTCTGTGTCGTCTTCTCTATATG-3′	400 nmol/l
Goose-121bp-P	5′-[FAM]-ACTCTGTTCAGCCTTGCGAAGACCTTATGC-[TAMRA][b]-3′	200 nmol/l

[a] PCR product = 113884 - ACGAGGATAG GTTGTGACAG CTGACTCTGT TCAGCCTTGC GAAGACCTTA TGCTGTCTAC AATTACCTAA TTGGAGGATA TAGAATTATA GAATCATATA GAGAAGACGA CACAGAGATT C - 114004 - NW_025927981.1.

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

Goose-121bp-F is base pairs 113 884 – 113 904, Goose-121bp-R is base pairs 113 978 – 114 404 and Goose-121bp-P is 113 907 – 113 936 of NW_025927981.1, *Anser cygnoides* domesticus unplaced genomic scaffold. 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 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 (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[a]	12,5 µl
Primer Goose-121bp-F, c = 10 µmol/l and Goose-121bp-R, c = 10 µmol/l	1,0 µl for each
Probe Goose-121bp-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 (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	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 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:

— goose-specific primers Goose-121bp-F and Goose-121bp-R and the probe Goose-121bp-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.

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 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.

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 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[3] and the BVL guidelines[4]. 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 goose 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.

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

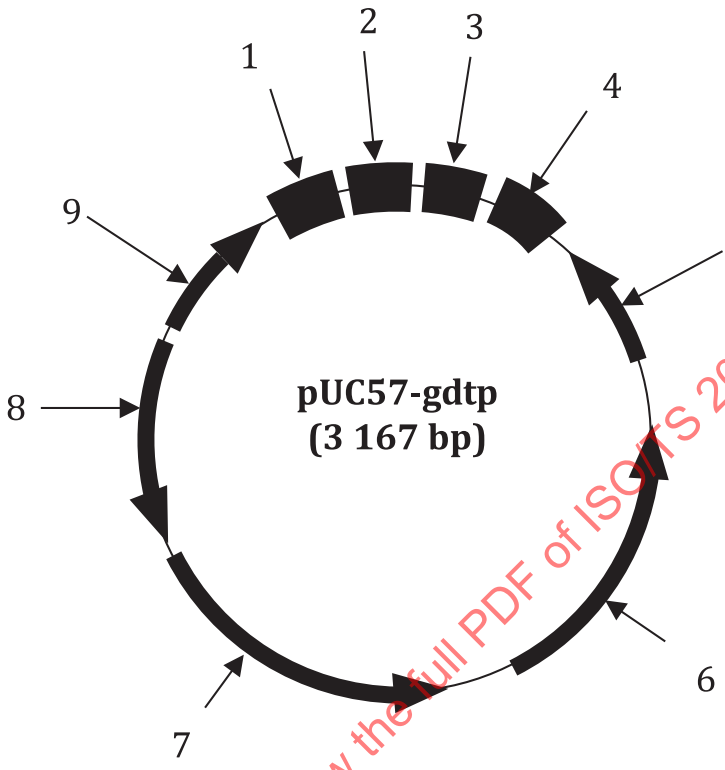
Table 4 — Results of the collaborative trial

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 goose material	72
Number of accepted samples not containing goose material	72
False-positive results	0
False-positive results (in %)	0
False-negative results	1
False-negative results (in %)	1,4

9.4 Sensitivity

The absolute limit of detection ($LOD_{95\ \%}$) for the method is five DNA copies. The collaborative trial of the goose detection method was carried out at the same time as collaborative trials for the duck, turkey and pigeon detection methods. Goose, duck, turkey and pigeon 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 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 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

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[5]). 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.[6]

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

Copy number of specific DNA sequence in goose genomic scaffold per PCR (nominal)	Number of positive results ($C_t < 45$) out of 72 results
20	72
10	72
5	72
2	68
1	57
0,5	38
0,1	16

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

Parameter		Specific DNA sequence in goose genomic scaffold
Number of laboratories		12
Number of PCR replicates per dilution level		6
POD curve	The mean probability of detection across laboratories (LPOD)	0,83
	95 % confidence interval for LPOD	0,78 to 0,87
	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 *Anser cygnoides* isolate SCWG-2014 breed Sichuan white goose unplaced genomic scaffold, GooseV1.0 scaffold320 (GenBank accession number NW_025927981.1) was selected as a PCR target.[2] Primers and probes were designed and optimized using primer-probe selection and optimization software.

The theoretical exclusive specificity of *Anser cygnoides domesticus* unplaced genomic scaffold sequence's primers and probes was analysed for homology to other species using the BLASTN program.[7] The 121-bp sequence used as query is part of the NCBI accession number NW_025927981.1 (nucleotides position: 113 884 – 114 004) 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.[7]

A 121 bp sequence equivalent to *Anser brachyrhynchus* (accession number NXHY01000202.1), *Anser indicus* (accession number VDDG01000056.1), *Branta canadensis* (accession number SNRU01005576.1), *Cygnus atratus* (accession number JABXOC010000147.1), *Cygnus buccinator* (accession number JAHMHU010001625.1), *Cygnus cygnus* (accession number BMBB01013197.1), *Cygnus olor* (accession number NC_049169.1) and *Nettapus auratus* (accession number JAACYP010002576.1), a 119 bp sequence equivalent to *Stictonetta naevosa* (accession number JAACYO010005739.1) and a 127 bp sequence equivalent to *Oxyura jamaicensis* (accession number NW_023310710.1) were synthesized and cloned into pUC57 separately for experimental testing in the absence of an available biological sample. Cross detection of *Anser brachyrhynchus*, *Anser indicus*, *Branta canadensis*, *Cygnus buccinator*, *Cygnus cygnus*, *Cygnus olor*, *Nettapus auritus*, *Stictonetta naevosa* and *Oxyura jamaicensis* of Anseriformes is expected.

The inclusive specificity was tested against nine breeds including *Anser cygnoides* domesticus Zhedong (China), Sichuan (China), Wanxi (China), Magang (China), Huoyan (China), Shitou (China), *Anser Anser* domesticus Carlos (Europe), White Roman (Italy) and Hortobagyi (Hungary). At approximate

100 copies of target DNA, all of the breed samples were detected with the expected positive signals and amplification curves.

Table 7 — Specificity of the goose unplaced genomic scaffold sequence detection method

Species test		Theoretically expected	Experimental confirmation
Anseriformes	*Anas platyrhynchos*	N	N
	Anas zonorhyncha	N	N
	Anser anser domesticus	Pos	Pos
	Anser brachyrhynchus	Pos	Pos[a]
	Anser cygnoides domesticus	Pos	Pos
	Anser indicus	Pos	Pos[b]
	Branta canadensis	Pos	Pos[c]
	Cairina moschata	N	N
	Cygnus atratus	Pos	Pos
	Cygnus buccinator	Pos	Pos[d]
	Cygnus cygnus	Pos	Pos[e]
	Cygnus olor	Pos	Pos[f]
	Nettapus auritus	Pos	Pos[g]
	Oxyura jamaicensis	Pos	Pos[h]
	Stictonetta naevosa	Pos	Pos[i]
Other animals	Turkey (*Meleagris gallopavo*)	N	N
	Chicken (*Gallus gallus*)	N	N
	Pheasant (*Phasianus colchicus*)	N	N
	Quail (*Coturnix coturnix*)	N	N
	Ostrich (*Struthio camelus*)	N	N
	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
	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
	Mouse (*Mus musculus*)	N	N
	Mule (*Equus caballus* × *asinus*)	N	N
	Pig (*Sus scrofa* domesticus)	N	N
	Pigeon (*Columba livia*)	N	N
	Rabbit (*Oryctolagus cunicul*us)	N	N
	Rat (*Rattus norvegicus*)	N	N
	Rhesus macaque (*Macaca mulatta*)	N	N
	Sheep (*Ovis aries*)	N	N

Table 7 *(continued)*

Species test		Theoretically expected	Experimental confirmation
	Trout (*Onchorhynchus mykiss*)	N	N
	Water buffalo (*Bubalus bubalis*)	N	N
	Yak (*Bos mutus*)	N	N
Human	Human (*Homo sapiens*)	N	N
Plant	Alfalfa (*Medicago sativa*)	N	N
	Corn (*Zea mays*)	N	N
	Rapeseed (*Brassica rapa*)	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

[a, b, c, d, e, f, g] In place of a biological sample of *Anser brachyrhynchus*, *Anser indicus*, *Branta canadensis*, *Cygnus buccinator*, *Cygnus cygnus*, *Cygnus olor* and *Nettapus auratus* for experimental testing, the high-homology 121 base target sequence of accession number NXHY01000202.1 (730 151 bp – 730 271 bp), VDDG01000056.1 (4 845 310 bp – 4 845 190 bp), SNRU01005576.1 (35 532 bp – 35 652 bp), JAHMHU010001625.1 (12 009 bp – 12 129 bp), BMBB01013197.1 (22 302 bp – 22 422 bp), NC_049169.1 (171 257 862 bp – 171 257 982 bp) and JAACYP010002576.1 (922 bp to 1 042 bp) identified by BALSTN was synthesized and cloned in plasmid pUC57.

[h] In place of a biological sample of *Oxyura jamaicensis* for experimental testing, the high-homology 127 base target sequence of accession number NW_023310710.1 (763 bp – 889 bp) identified by BALSTN was synthetized and cloned in plasmid pUC57.

[i] In place of a biological sample of *Stictonetta naevosa* for experimental testing, the high-homology 119 base target sequence of accession number JAACYO010005739.1 (770 bp – 888 bp) identified by BALSTN 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_025927981.1 (113 884 – 114 004 bp).

A.1.2 Description: *Anser cygnoides* isolate SCWG-2014 breed Sichuan white goose unplaced genomic scaffold, GooseV1.0 scaffold320.

A.1.3 Molecule type: nucleic acid.

A.1.4 Query length: 121 bp.

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
Anser cygnoides isolate SCWG-2014 breed Sichuan white goose unplaced genomic scaffold, GooseV1.0 scaffold320	224	224	100	2e-55	100,00	NW_025927981.1	611258	GenBank RefSeq Genome (refseq_genomes)	Anseriformes (taxid:8826)
Anser indicus isolate heart tissue scaffold_1388, whole genome shotgun sequence	224	224	100	3e-55	100,00	VDDG01000056.1	11358879	Whole-genome Shotgun Contigs (wgs)	Anseriformes (taxid:8826)
Anser brachyrhynchus strain PFG001 scaffold_201, whole genome shotgun sequence	224	224	100	3e-55	100,00	NXHY01000202.1	1368934	Whole-genome Shotgun Contigs (wgs)	Anseriformes (taxid:8826)
Branta canadensis isolate SJ_97 contig25_131, whole genome shotgun sequence	219	219	100	1e-53	99,17	SNRU01005576.1	59448	Whole-genome Shotgun Contigs (wgs)	Anseriformes (taxid:8826)
Cygnus buccinator isolate WWS_Y101 seq_1625, whole genome shotgun sequence	213	213	100	6e-52	98,35	JAHMHU010001625.1	30693	Whole-genome Shotgun Contigs (wgs)	Anseriformes (taxid:8826)

Table A.1 *(continued)*

Description	Max. score	Total score	Query cover %	E value	% Ident	Accession	Accession length	Standard databases	Organism
Cygnus atratus isolate AKBS03 ecotype Queensland, Australia unplaced genomic scaffold, Cygnus_atratus_primary_v1.0 000174F	213	213	100	5e-52	98,35	NW_023336758.1	654044	GenBank RefSeq Genome (refseq_genomes)	Anseriformes (taxid:8826)
Cygnus cygnus DNA, Cycy_13197, whole genome shotgun sequence	213	213	100	6e-52	98,35	BMBB01013197.1	23417	Whole-genome Shotgun Contigs (wgs)	Anseriformes (taxid:8826)
Cygnus olor isolate bCygOlo1 chromosome 1, bCygOlo1.pri.v2	207	207	100	2e-50	97,52	NC_049169.1	209522193	GenBank RefSeq Genome (refseq_genomes)	Anseriformes (taxid:8826)
Stictonetta naevosa breed freckled duck isolate SHBPF4130 SN47.24, whole genome shotgun sequence	180	180	98	6e-42	94,12	JAACYO010005739.1	33151	Whole-genome Shotgun Contigs (wgs)	Anseriformes (taxid:8826)
Nettapus auritus breed African pygmy goose isolate SHBP007 NA37.712, whole genome shotgun sequence	171	171	98	3e-39	92,44	JAACYP010002576.1	35822	Whole-genome Shotgun Contigs (wgs)	Anseriformes (taxid:8826)
Oxyura jamaicensis isolate SHBP4307 breed ruddy duck unplaced genomic scaffold, BPBGC_Ojam_1.0 oxyUn_random_OJ71826	154	154	98	3e-34	89,60	NW_023310710.1	10665	GenBank RefSeq Genome (refseq_genomes)	Anseriformes (taxid:8826)

A.3 Alignments

Anser cygnoides isolate SCWG-2014 breed Sichuan white goose unplaced genomic scaffold, GooseV1.0 scaffold320, Sequence ID: NW_025927981.1

Length: 1496546 Number of matches: 1 Range 1: 113 884 to 114 004

Score	Expect	Identities	Gaps	Strand
224bits(121)	2e-55	121/121(100 %)	0/121(0 %)	Plus/Plus

```
Query  1       ACGAGGATAGGTTGTGACAGCTGACTCTGTTCAGCCTTGCGAAGACCTTATGCTGTCTAC  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  113884  ACGAGGATAGGTTGTGACAGCTGACTCTGTTCAGCCTTGCGAAGACCTTATGCTGTCTAC  113943

Query  61      AATTACCTAATTGGAGGATATAGAATTATAGAATCATATAGAGAAGACGACACAGAGATT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  113944  AATTACCTAATTGGAGGATATAGAATTATAGAATCATATAGAGAAGACGACACAGAGATT  114003

Query  121     C 121
               |
Sbjct  114004  C 114004
```

Anser indicus isolate heart tissue scaffold_1388, whole genome shotgun sequence

Sequence ID: VDDG01000056.1

Length: 11358879 Number of matches: 1 Range 1: 4845310 to 4845190

Score	Expect	Identities	Gaps	Strand
224 bits(121)	3e-55	121/121(100 %)	0/121(0 %)	Plus/Minus

```
Query  1         ACGAGGATAGGTTGTGACAGCTGACTCTGTTCAGCCTTGCGAAGACCTTATGCTGTCTAC  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4845310   ACGAGGATAGGTTGTGACAGCTGACTCTGTTCAGCCTTGCGAAGACCTTATGCTGTCTAC  4845251

Query  61        AATTACCTAATTGGAGGATATAGAATTATAGAATCATATAGAGAAGACGACACAGAGATT  120
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4845250   AATTACCTAATTGGAGGATATAGAATTATAGAATCATATAGAGAAGACGACACAGAGATT  4845191

Query  121       C 121
                 |
Sbjct  4845190   C 4845190
```

Anser brachyrhynchus strain PFG001 scaffold_201, whole genome shotgun sequence

Sequence ID: NXHY01000202.1

Length: 1368934 Number of matches: 1 Range 1: 730151 to 730271

Score	Expect	Identities	Gaps	Strand
224 bits(121)	3e-55	121/121(100 %)	0/121(0 %)	Plus/Plus

```
Query  1         ACGAGGATAGGTTGTGACAGCTGACTCTGTTCAGCCTTGCGAAGACCTTATGCTGTCTAC  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  730151    ACGAGGATAGGTTGTGACAGCTGACTCTGTTCAGCCTTGCGAAGACCTTATGCTGTCTAC  730210

Query  61        AATTACCTAATTGGAGGATATAGAATTATAGAATCATATAGAGAAGACGACACAGAGATT  120
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  730211    AATTACCTAATTGGAGGATATAGAATTATAGAATCATATAGAGAAGACGACACAGAGATT  730270

Query  121       C 121
                 |
Sbjct  730271    C 730271
```