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

## Part 10:
## Duck 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 10: Méthode de détection de l'ADN de canard*

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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 mallard duck (*Anas platyrhynchos*) and spot-billed duck (*Anas zonorhyncha*) DNA detection. Cross detection of white-winged duck (*Asarcornis scutulata*), tufted duck (*Aythya fuligula*), muscovy duck (*Cairina moschata*) and Mandarin duck (*Aix galericulata*) is observed.

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 10:
## Duck DNA detection method

## 1 Scope

This document specifies a real-time polymerase chain reaction (real-time PCR) method for the qualitative detection of duck-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 duck material derived from mallard duck (*Anas platyrhynchos*) and spot-billed duck (*Anas zonorhyncha*). Cross detection of white-winged duck (*Asarcornis scutulata*), tufted duck (*Aythya fuligula*), muscovy duck (*Cairina moschata*) and Mandarin duck (*Aix galericulata*) is observed. Mallard duck and muscovy duck are domesticated poultry for food, while spot-billed duck, white-winged duck, tufted duck and Mandarin duck are wild avian.

The target sequence is a partial fragment of the *Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence (i.e. GenBank accession number JACEUM010000001.1),[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 duck species-specific DNA sequence of the single-copy *Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence (i.e. GenBank accession number JACEUM010000001.1) 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 duck genome and myostatin gene in mammals and poultry genome are single copy. The copy number of the specific target sequence in *Anas platyrhynchos* 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.

NOTE The commercial real-time PCR master mix can 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 *Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence (i.e. GenBank accession number JACEUM010000001.1)[a] | | |
| Duck-105bp-F | 5′-TCTTCACAAGCAGGGTCTAATGG-3′ | 400 nmol/l |
| Duck-105bp-R | 5′-CTTGGCAGAAGGTCCAGAGG-3′ | 400 nmol/l |
| Duck-105bp-P | 5′- [FAM]-AGGCACAGCACGCATCTCACCACA-[TAMRA][b]-3′ | 200 nmol/l |

[a] PCR product = 200 064 152 - TCTTCACAAG CAGGGTCTAA TGGAAGACTT GCTGGCCTGC TCTCTACTGG TGATGATGTG GTGAGATGCG TGCTGTGCCT CTCTTCCTCT GGACCTTCTG CCAAG - 200 064 048 - JACEUM010000001.1

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

Duck-105bp-F is base pairs 200 064 152 – 200 064 130, Duck-105bp-R is base pairs 200 064 048 – 200 064 067 and Duck-105bp-P is 200 064 073 – 200 064 096 of JACEUM010000001.1, *Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence. Equivalent reporter dyes and/or quencher dyes can be used if they yield the same or better results.

## 6 Apparatus

Requirements concerning apparatus and materials shall follow ISO 20813. In addition to the usual laboratory equipment, the following equipment is required.

### 6.1 Real-time thermocycler instrument.

A device that amplifies DNA *in vitro* and performs the temperature-time cycles is needed for PCR. Additionally, the device shall be capable of exciting fluorescence molecules at specific wavelengths and detecting 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 Duck-105bp-F, c = 10 µmol/l and Duck-105bp-R, c = 10 µmol/l | 1,0 µl for each |
| Probe Duck-105bp-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 can 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:

- duck-specific primers Duck-105bp-F and Duck-105bp-R and the probe Duck-105bp-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]);

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[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;

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 duck 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.

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

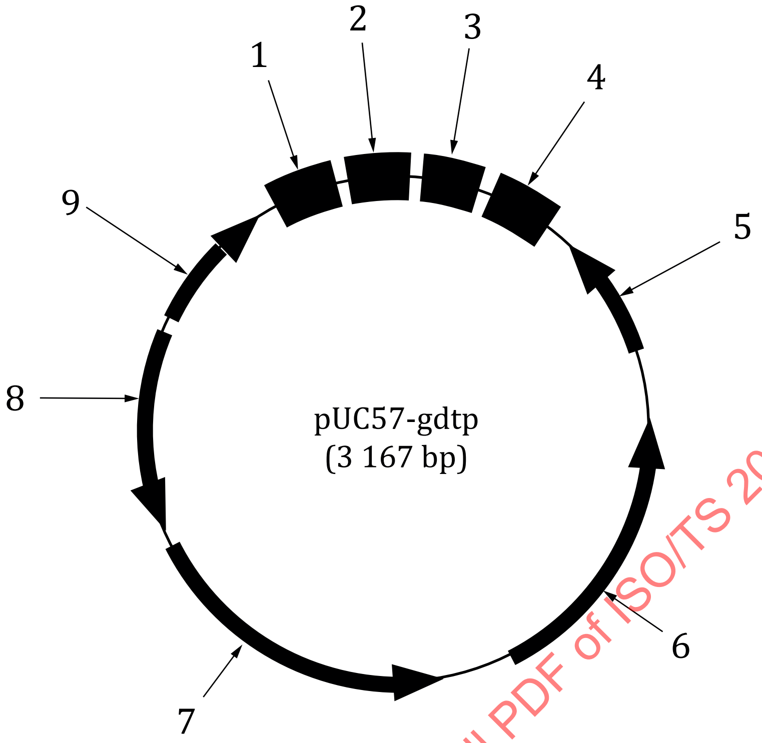
The collaborative trial was designed to determine false-positive and false-negative rates. Each DNA sample was tested by the participants in a single PCR test with 5 µl of the respective DNA solution, using the procedure and the conditions given in Tables 2 and 3. The results of the collaborative trial 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 duck material | 72 |
| Number of accepted samples not containing duck 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 duck detection method was carried out at the same time as collaborative trials for the goose, turkey, and pigeon 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 0,30 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 duck genomic sequence per PCR (nominal) | Number of positive results ($C_t$ < 45) out of 72 results |
|---|---|
| 20 | 72 |
| 10 | 72 |
| 5 | 72 |
| 2 | 68 |
| 1 | 49 |
| 0,5 | 34 |
| 0,1 | 8 |

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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 duck genomic sequence |
|---|---|---|
| Number of laboratories | | 12 |
| Number of PCR replicates per dilution level | | 6 |
| POD curve | The mean probability of detection across laboratories (LPOD) | 0,80 |
| | 95 % confidence interval for LPOD | 0,76 to 0,83 |
| | Slope b relative to the ideal POD curve (b = 1) | 1,16 |
| | Laboratory standard deviation, $\sigma_L$ | 0,30 |
| $LOD_{95\,\%}$ (in copies) | Theoretical median laboratory | 3,1 |

## 9.5 Specificity

A representative sequence from the *Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence (i.e. GenBank accession number JACEUM010000001.1) was selected as a PCR target according to the alignment results among reference genomes of various bird species at the beginning of 2019.[1] Primers and probes were designed and optimized using primer-probe selection and optimization software.

The theoretical exclusive specificity of *Anas platyrhynchos* genome sequence's primers and probes was analysed for homology to other species using the BLASTN program.[6] The 105-bp sequence used as query is part of the NCBI accession number JACEUM010000001.1 (nucleotides position: 200 064 152 – 200 064 048). Similarity search results are given in Annex A. There was no homology with other genes and species.

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].

This PCR amplification is specific to mallard duck (*Anas platyrhynchos*) and spot-billed duck (*Anas zonorhyncha*) DNA detection as expected. Further experimental tests observed cross detection of Mandarin duck (*Aix galericulata*), white-winged duck (*Asarcornis scutulata*), tufted duck (*Aythya fuligula*) and muscovy ducks (*Cairina moschata*).

The inclusive specificity was tested against nine breeds including *Anas platyrhynchos* Common Mallard (unknown origin, egg and meat), Khaki Campbell (England, egg), Cherry Valley (England, meat), Pekin-duck (China, meat), Tegel (Australia, meat), Shaoxing (China, egg), Jinding (China, egg), Gaoyou (China, egg and meat) and *Anas zonorhyncha* breed spot-billed (China, wild). 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 105 base target sequence was also evaluated using the BLASTN program against the GenBank whole animal genomes database. Results indicating that the 105 base target sequence is unique for duck are provided in Annex A.

Members of the *Anatidae* family[7] and its family tree established with available public genomic sequences are shown in Figure B.1.

**Table 7 — Specificity of the target duck genomic sequence detection method**

| Species test | | Theoretically expected | Experimental confirmation |
|---|---|---|---|
| **Animal** | Bison (*Bison bison*) | N | N |
| | Black swan (*Cygnus atratus*) | 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 |
| | Mallard duck (*Anas platyrhynchos*) | Pos | Pos |
| | Mandarin duck (*Aix galericulata*) | Pos | Pos |
| | Mouse (*Mus musculus*) | N | N |
| | Muscovy duck (*Cairina moschata*) | Pos | Pos |
| | 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*) | N | N |
| | Sheep (*Ovis aries*) | N | N |
| | Eastern spot-billed duck (*Anas zonorhyncha*) | Pos | Pos |
| | Swan goose (*Anser cygnoides* domesticus) | N | N |
| | Tufted duck (*Aythya fuligula*) | Pos | Pos[a] |
| | Eastern spotted dove (*Streptopelia chinensis*) | N | N |
| | Water buffalo (*Bubalus bubalis*) | N | N |
| | Wild turkey (*Meleagris Ocellata*) | N | N |
| | White-winged duck (*Asarcornis scutulata*) | Pos | Pos[b] |

**Key**

Pos: positive; N: negative

[a] In place of a biological sample of Tufted duck (*Aythya fuligula*) for experimental testing, the high-homology 105 base target sequence of accession number WNMM01000001.1 (199 307 524 bp – 199 307 628 bp) identified by BALSTN was synthesized and cloned in plasmid pUC57.

[b] In place of a biological sample of White-winged duck (*Asarcornis scutulata*) for experimental testing, the high-homology 105 base target sequence of accession number VZSO01008967.1 (752 bp – 648 bp) identified by BALSTN was synthesized and cloned in plasmid pUC57.

**Table 7** *(continued)*

| Species test | | Theoretically expected | Experimental confirmation |
|---|---|---|---|
| | Wild yak (*Bos mutus*) | N | N |
| **Human** | Human (*Homo sapiens*) | N | N |
| **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

[a] In place of a biological sample of Tufted duck (*Aythya fuligula*) for experimental testing, the high-homology 105 base target sequence of accession number WNMM01000001.1 (199 307 524 bp – 199 307 628 bp) identified by BALSTN was synthesized and cloned in plasmid pUC57.

[b] In place of a biological sample of White-winged duck (*Asarcornis scutulata*) for experimental testing, the high-homology 105 base target sequence of accession number VZSO01008967.1 (752 bp – 648 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:** JACEUM010000001.1 (200 064 152 to 200 064 048 bp).

**A.1.2 Description:** *Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence.

**A.1.3 Molecule type:** nucleic acid.

**A.1.4 Query length:** 105 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 |
|---|---|---|---|---|---|---|---|---|---|
| *Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence | 195 | 195 | 100 | 2e-47 | 100 | JACEUM010000001 | 207246783 | Whole-genome Shotgun Contigs (wgs) | birds (taxid: 8782) |
| *Anas zonorhyncha* breed spot-billed scaffold627, whole genome shotgun sequence | 195 | 195 | 100 | 2e-47 | 100 | NOIK01000625.1 | 360378 | Whole-genome Shotgun Contigs (wgs) | birds (taxid: 8782) |
| *Asarcornis scutulata* isolate OUT-0051 scaffold113644, whole genome shotgun sequence | 191 | 191 | 98 | 3e-46 | 100 | VZSO01008967.1 | 1936 | Whole-genome Shotgun Contigs (wgs) | birds (taxid: 8782) |
| *Aythya fuligula* isolate bAytFul2 chromosome 1, bAytFul2.pri | 185 | 185 | 98 | 9e-44 | 99 | NC_045559.1 | 207018403 | GenBank RefSeq Genome (refseq_genomes) | birds (taxid: 8782) |
| *Aix galericulata* isolate NCHU-2012 Aplatyrhynchos.1_RagTag, whole genome shotgun sequence | 180 | 180 | 98 | 6e-43 | 98,06 | JALLAJ010000001.1 | 201074787 | Whole-genome Shotgun Contigs (wgs) | birds (taxid: 8782) |
| *Cairina moschata domestica* isolate CanardBarbarie3_02_2015_G Scaffold136, whole genome shotgun sequence | 169 | 169 | 98 | 1e-39 | 96,12 | QZEJ01000136.1 | 2119812 | Whole-genome Shotgun Contigs (wgs) | birds (taxid: 8782) |

## A.3 Alignments

*Anas platyrhynchos* breed pekin duck isolate CAU_Pekin_2.0 Chr1, whole genome shotgun sequence

Sequence ID: JACEUM010000001

Length: 207246783 Number of matches: 1 Range 1: 200064048 to 200064152

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 195 bits(105) | 2e-47 | 105/105(100 %) | 0/105(0 %) | Plus/Minus |

```
Query  1          TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  200064152  TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  200064093

Query  61         GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG  105
                  |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  200064092  GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG  200064048
```

*Anas zonorhyncha* breed spot-billed scaffold627, whole genome shotgun sequence

Sequence ID: NOIK01000625.1

Length: 360378 Number of matches: 1 Range 1: 51915 to 52019

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 195 bits(105) | 2e-47 | 105/105(100 %) | 0/105(0 %) | Plus/Minus |

```
Query  1      TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  52019  TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  51960

Query  61     GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG  105
              |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  51959  GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG  51915
```

*Asarcornis scutulata* isolate OUT-0051 scaffold113644, whole genome shotgun sequence

Sequence ID: VZSO01008967.1

Length: 1936 Number of matches: 1 Range 1: 648 to 752

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 191 bits(103) | 3e-46 | 103/103(100 %) | 0/103(0 %) | Plus/Minus |

```
Query  1     TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  752   TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  693

Query  61    GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG  105
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  692   GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAGG  648
```

*Aythya fuligula* isolate bAytFul2 chromosome 1, whole genome shotgun sequence

Sequence ID: WNMM01000001.1

Length: 207018403 Number of matches: 1 Range 1: 199307524 to 199307628

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 185 bits(100) | 1e-44 | 102/103(99 %) | 0/103(0 %) | Plus/Minus |

```
Query  1          TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199307628  TCTTCACAAGCAGGGTCTAATGAAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  199307569

Query  61         GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG  105
                  |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199307568  GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAGG  199307524
```

*Aix galericulata* isolate NCHU-2012 Aplatyrhynchos.1_RagTag, whole genome shotgun sequence

Sequence ID: JALLAJ010000001.1

Length: 201074787 Number of matches: 1 Range 1: 193845780 to 193845884

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 180 bits(97) | 6e-43 | 101/103(98 %) | 0/103(0 %) | Plus/Minus |

```
Query  1          TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG  60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193845884  TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTGCTGGTGATGATGTG  193845825

Query  61         GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG  105
                  |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193845824  GTGAGATGCGTGCCGTGCCTCTCTTCCTCTGGACCTTCTGCCAGG  193845780
```

*Cairina moschata* domestica isolate Canard Barbarie3_02_2015_G Scaffold136, whole genome shotgun sequence

Sequence ID: QZEJ01000136.1

Length: 2119812 Number of matches: 1 Range 1: 1872455 to 1872559

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 169 bits(91) | 1e-39 | 99/103(96 %) | 0/103(0 %) | Plus/Minus |

```
Query   1         TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTACTGGTGATGATGTG   60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1872559   TCTTCACAAGCAGGGTCTAATGGAAGACTTGCTGGCCTGCTCTCTGCTGCTGATGACGTG   1872500

Query   61        GTGAGATGCGTGCTGTGCCTCTCTTCCTCTGGACCTTCTGCCAAG   105
                  |||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1872499   GTGAGATGCGTGCCGTGCCTCTCTTCCTCTGGACCTTCTGCCAGG   1872455
```

# Annex B
(informative)

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

## B.1 Members of the *Anatidae* family

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

| Genus No. | Genus | Species | Accession number of available public genomic sequence |
|---|---|---|---|
| 1 | *Aix* | *galericulata* | JALLAJ000000000.1 (wgs[a]) |
| | | *sponsa* | |
| 2 | *Alopochen* | *aegyptiaca* | |
| | | *kervazoi* | |
| | | *mauritiana* | |
| 3 | *Amazonetta* | *brasiliensis* | |

[a] wgs: whole genome shotgun

**Table B.1** *(continued)*

| Genus No. | Genus | Species | Accession number of available public genomic sequence |
|---|---|---|---|
| 4 | *Anas* | *acuta* | |
| | | *albogularis* | |
| | | *andium* | |
| | | *aucklandica* | |
| | | *bahamensis* | |
| | | *bernieri* | |
| | | *capensis* | |
| | | *carolinensis* | |
| | | *castanea* | |
| | | *chlorotis* | |
| | | *crecca* | |
| | | *diazi* | |
| | | *eatoni* | |
| | | *erythrorhyncha* | |
| | | *flavirostris* | |
| | | *fulvigula* | |
| | | *georgica* | |
| | | *gibberifrons* | |
| | | *gracilis* | |
| | | *laysanensis* | |
| | | *luzonica* | |
| | | *melleri* | |
| | | *nesiotis* | |
| | | *platyrhynchos* | JACEUM000000000.1 (wgs) |
| | | *poecilorhyncha* | |
| | | *rubripes* | |
| | | *sparsa* | |
| | | *superciliosa* | |
| | | *theodori* | |
| | | *undulata* | |
| | | *wyvilliana* | |
| | | *zonorhyncha* | NOIK01000625.1 (wgs) |

[a] wgs: whole genome shotgun

**Table B.1** *(continued)*

| **Genus No.** | **Genus** | **Species** | **Accession number of available public genomic sequence** |
|---|---|---|---|
| 5 | *Anser* | *albifrons* | |
| | | *anser* | |
| | | *brachyrhynchus* | NXHY01000202.1 (wgs) |
| | | *caerulescens* | |
| | | *canagicus* | |
| | | *cygnoides* | NW_025927981.1 (genome) |
| | | *erythropus* | |
| | | *fabalis* | |
| | | *indicus* | VDDG01000056.1 (wgs) |
| | | *rossii* | |
| | | *serrirostris* | |
| 6 | *Asarcornis* | *scutulata* | VZSO01008967.1 (wgs) |
| 7 | *Aythya* | *affinis* | |
| | | *australis* | |
| | | *baeri* | JAKRSJ000000000.1 ( (wgs) |
| | | *collaris* | |
| | | *ferina* | |
| | | *fuligula* | NC_045559.1 (genome) |
| | | *innotata* | |
| | | *marila* | JAOQIG000000000.1 (wgs) |
| | | *novaeseelandiae* | |
| | | *nyroca* | |
| | | *valisineria* | |
| 8 | *Biziura* | *lobata* | |
| 9 | *Branta* | *bernicla* | |
| | | *canadensis* | SNRU01005576.1 (wgs) |
| | | *hutchinsii* | |
| | | *leucopsis* | |
| | | *ruficollis* | |
| | | *sandvicensis* | |
| 10 | *Bucephala* | *albeola* | |
| | | *clangula* | |
| | | *islandica* | |
| 11 | *Cairina* | *moschata* | QZEJ01000136.1 (wgs) |
| 12 | *Callonetta* | *leucophrys* | |
| 13 | *Camptorhynchus* | *labradorius* | |
| 14 | *Cereopsis* | *novaehollandiae* | |
| 15 | *Chloephaga* | *hybrida* | |
| | | *melanoptera* | |
| | | *picta* | |
| | | *poliocephala* | |
| | | *rubidiceps* | |

[a] wgs: whole genome shotgun