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

Part 4: Chicken 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 4: Méthode de détection de l'ADN de poulet

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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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 and interpretation of results, specific to chicken 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 4: Chicken DNA detection method

1 Scope

This document specifies a real-time polymerase chain reaction (real-time PCR) method for the qualitative detection of chicken-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 chicken material derived from chicken (*Gallus gallus domesticus*) and jungle fowl (*Gallus gallus*).

The target sequence is a partial fragment of the *Gallus gallus* transforming growth factor beta 3, intron 4 (TGF-β3) gene (i.e. GenBank accession number AY685072.1)[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 % replicability 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 — Terms and definitions*

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 terminological databases for use in standardization at the following addresses:

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

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

— detection of the chicken species-specific DNA sequence of the single-copy TGF-β3 gene (GenBank accession number AY685072.1) 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 TGF-β3 gene in the chicken genome and myostatin gene in mammals and poultry genome are single copy. The copy number of the TGF-β3 gene in the chicken 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 chemicals 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.

PCR master mix contains thermostable DNA polymerase, pH buffer, KCl, $MgCl_2$, uracil-DNA glycosylase (UDG) and the four dNTPS (dATP, dGTP, dUTP, dCTP) as a dilutable concentrate, which 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
Chicken TGF-β3 gene as the target sequence (GenBank accession number AY685072.1)[a]		
Chicken-77bp-F	5′-CAGCTGGCCTGCCGGC-3′	400 nmol/l
Chicken-77bp-R	5′-GCCCAGTGGAATGTGGTATTCA-3′	400 nmol/l
Chicken-77bp-P	5′-[FAM]-TGCCACTCCTCTGCACCCAGTGC-[TAMRA][b]-3′	200 nmol/l

[a] PCR product = 313 - CAGCTGGCCT GCCGGCTTCT GCCAAGCTCT GCCACTCCTC TGCACCCAGT GCAGGTGAAT ACCACATTCC ACTGGGC - 389 - AY685072.1.

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

Chicken-77bp-F is base pairs 313-328, Chicken-77bp-R is base pairs 368-389 and Chicken-77bp-P is 342-364 of AY685072.1, chicken TGF-β3 gene. 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 Chicken-77bp-F, c = 10 µmol/l and Chicken-77bp-R, c = 10 µmol/l	1,0 µl for each
Probe Chicken-77bp-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 (e.g. 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:

— chicken-specific primers Chicken-77bp-F and Chicken-77bp-R and the probe Chicken-77bp-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]);

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

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.

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 13 participants, organized by the Technical Center for Animal, Plant and Food Inspection and Quarantine, Shanghai Customs[2] 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 chicken DNA solution, 10 copies/µl;

— six vials of horse 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.

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

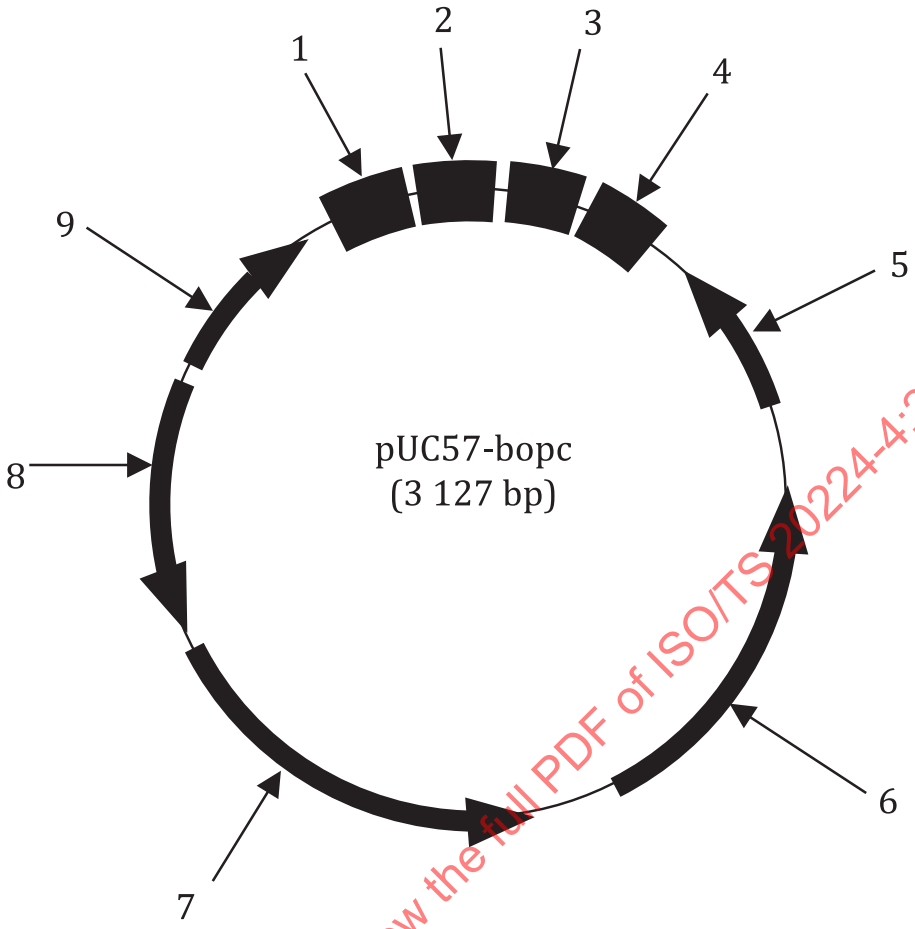
The collaborative trial was designed to determine false-positive and false-negative rates. Each DNA sample was tested by the participants in a single PCR test with 5 µl of the respective DNA solution, using the procedure and the conditions given in Tables 2 and 3. The results of the collaborative trial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

Table 4 — Results of the collaborative trial

Number of laboratories	13
Number of laboratories submitting results	13
Number of samples per laboratory	12
Number of accepted results	156
Number of accepted samples containing chicken material	78
Number of accepted samples not containing chicken material	78
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 chicken detection method was carried out at the same time as collaborative trials for the bovine, porcine and ovine detection methods. Bovine, ovine, porcine and chicken 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-bopc (3 127 bp in length) was sequenced to ensure that only one copy of the bovine, ovine, porcine and chicken 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 1–62 = bovine amplicon (62 bp)
2 nt 63~150 = ovine amplicon (88 bp)
3 nt 244~340 = porcine amplicon (97 bp)
4 nt 341~417 = chicken amplicon (77 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 GGCCTCGGAG TGTGTATTCA GTAGGTGCAC AGTACGTTCT GAAGTGAACC

51 TCATTCTGGG GCCCAACATG CCTTTAAACC CTCAAAAACC ATTGAGACTG

101 GCGGGGAAGG AAAGGCAGCC AAACAGAGCG AGTCAGAAGG CTACAGTTCC

151 acacaatgtt acgcgtatgc aagtacatta caccgctcgc ctacacacaa

201 atacatttac taacatccat ataacgcgga catacagcct tcaCGTAGGT

251 GCACAGTAGG TCTGACGTGA CTCCCCGACC TGGGGTCCCC AGCACACTTA

301 GCCGTGTTCC TTGCACTCTC TGCATGTCCC CAGTCTGGCC CAGCTGGCCT

351 GCCGGCTTCT GCCAAGCTCT GCCACTCCTC TGCACCCAGT GCAGGTGAAT

401 ACCACATTCC ACTGGGC

Key

single bold underline	nt 1~62 = bovine amplicon (62 bp)
double underline	nt 63~150 = ovine amplicon (88 bp)
dashed underline	nt 244~340 = porcine amplicon (97 bp)
dotted underline	nt 341~417 = chicken amplicon (77 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 Reference [2] and 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 13 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 78 out of 78 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. 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 [4]. Standard deviation was determined to be 0,30 and the $LOD_{95\ \%}$ was 3,3 copies; both parameters well below the required maximum of 1 and 20 copies, respectively[2][5].

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 ($LOD_{95\ \%}$)

Chicken TGF-β3 gene copy number per PCR (nominal)	Number of positive results ($C_t < 45$) out of 78 results
20	78
10	78
5	78
2	69
1	42
0,5	25
0,1	6

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

Parameter		Chicken TGF-β3 gene
Number of laboratories		13
Number of PCR replicates per dilution level		6
POD curve	The mean probability of detection across laboratories (LPOD)	0,74
	Slope b relative to the ideal POD curve (b = 1)	1,18
	Laboratory standard deviation, σ_L	0,30
$LOD_{95\ \%}$ (in copies)	Theoretical median laboratory	3,3

9.5 Specificity

A representative sequence from the chicken TGF-β3 gene (GenBank accession number AY685072.1) was selected as a PCR target[1]. Primers and probes were designed and optimized using primer-probe selection and optimization software.

The theoretical exclusive specificity of chicken TGF-β3 gene primers and probes was analysed for homology to other species using the BLASTN program[6]. The 77-bp sequence used as query is part of the NCBI accession number AY685072.1 (nucleotides position: 313-389). 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].

The inclusive specificity was tested against 10 breeds including *Gallus gallus* Roman Hens (Germany, egg), White Leghorns (Italy, egg), White Plymouth rock (USA, meat), Ross (England, egg), Langshan (China, meat and egg), Luyuan (China, meat and egg), Xiaoshan (China, meat and egg), Yangshan (China, meat), White Recessive Rocks (France, meat) and *Gallus gallus* Jabouillei (Jungle fowl, China, meat). At approximate 100 copies of target DNA, all the breed samples were detected with the expected positive signals and amplification curves. Inclusivity of the 77 base target sequence was also evaluated using the BLASTN program against the GenBank whole animal genomes database. Results indicating that the 77 base target sequence is unique for chicken animals are provided in Annex A.

Table 7 — Specificity of the chicken TGF-β3 gene 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* domesticus)	Pos	Pos
	Dog (*Canis familiaris*)	N	N
	Donkey (*Equus asinus*)	N	N
	Duck (*Anas platyrhynchos*)	N	N
	Elk (*Cervus canadensis*)	N	N
	Goat (*Capra hircus*)	N	N
	Goldfish (*Carassius auratus*)	N	N
	Goose (*Anser anser*)	N	N
	Horse (*Equus caballus*)	N	N
	Indian zebu (*Bos indicus*)	N	N
	Jungle fowl (*Gallus gallus*)	Pos	Pos
	Mouse (*Mus musculus*)	N	N
	Rhesus macaque (*Macaca mulatta*)	N	N
	Ostrich (*Struthio camelus*)	N	N
	Pheasant (*Phasianus colchicus*)	N	N
	Pig (*Sus scrofa* domesticus)	N	N
	Pigeon (*Columba livia*)	N	N
	Quail (*Coturnix coturnix*)	N	N
	Rabbit (*Oryctolagus cuniculus*)	N	N
	Rat (*Rattus norvegicus*)	N	N
	Sheep (*Ovis aries*)	N	N
	Trout (*Onchorhynchus mykiss*)	N	N
	Turkey (*Meleagris gallopavo*)	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

10 Test report

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