# Combined microscopy-PCR
# EURL-AP Proficiency Test 2025

## *Final version*

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# Summary

The European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP) organised the present proficiency test for assessing the ability of the NRL network with respect to the detection of processed animal proteins (PAPs) in feed using both light microscopy and PCR according to current legal requirements.

The total number of registered laboratories was 32 (26 NRLs and 6 labs outside the NRL network). The study was based on a set of five samples (to be analysed both by light microscopy and PCR) consisting of blank feed matrices or feed materials fortified or not with processed animal proteins from terrestrial vertebrates, terrestrial invertebrates and/or from fish.

The results of two NRLs do not appear in this report. One NRL sent its results after the deadline and the other laboratory sent only partial results which cannot be accepted.

Results from light microscopy showed that 58 % of NRLs achieved an excellent score, while 2 % obtained satisfactory scores. In contrast, 34 % of NRLs were classified as underperforming. This high rate of underperformance is mainly linked to the laboratories' difficulty in identifying lactose crystals. A second source of difficulty was the failure to detect poultry bones within a fish meal. This well-known masking effect, caused by a large proportion of fish fragments, should alert participants to the need for greater attention to other constituents when such a matrix is used.

Concerning the PCR results, participants were assessed on their proficiency to perform the three PCR tests for the detection of ruminant, pig and poultry DNA.   81 % of the NRLs (21 out of 26) performed excellently and reported no false result. Three NRLs (11 %) were considered as underperforming: two of them recorded one deviation for the detection of ruminant DNA whereas the remaining lab cumulated two false results for the detection of pig DNA. Results of the two last NRLs were not considered in this report. The overall performance of the network remains excellent, although slightly lower than in 2024, with the three PCR methods. With a total of four deviations out of the 336 PCR results recorded, the global accuracy of the network is 0.988.

*This report identified by an ISBN has been prepared from a draft version sent for revision and comments to the participants on the 2nd February 2026.  After reception and evaluation of the comments on the 23rd February 2026, it was amended accordingly and approved by the signature of the organisers.*

*ISO 17043 coordinators signature for approval:*

*Olivier Fumière*
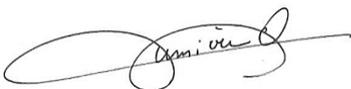
*Pascal Veys*

# 1. Foreword

European Union Reference Laboratories (EURLs) were created to ensure a high level of quality and a uniformity of the results provided by European control laboratories. On 15th March 2017, the European Parliament and the Council adopted Regulation EU/625/2017 [1], improving the effectiveness of the official food and feed controls while redefining the obligations of the relevant authorities and their obligations in the organization of these controls.

On March 2011, Commission Regulation EC/208/2011 [2] renewed the nomination of the Walloon Agricultural Research Centre as European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP, https://www.eurl.craw.eu). It has to develop the following priority axes:

(i) To provide National Reference Laboratories (NRLs) with detailed analytical methods, including reference methods for the network of Member State NRLs;

(ii) To coordinate application by NRLs of the methods by organising interlaboratory studies;

(iii) To develop new analytical methods for the detection of animal proteins in feedingstuffs (light microscopy, near infrared microscopy, PCR, immunology, …);

(iv) To conduct training courses for the benefit of NRL staffs from Member States and future Member States;

(v) To provide scientific and technical assistance to the European Commission, especially in cases of disputed results between Member States.

In this framework, the EURL-AP has been organising yearly since 2006 proficiency tests for the assessment of the implementation of the reference methods for the detection of animal proteins in feed as described by current Annex VI of Commission Regulation EC/152/2009 [3]. Since 2016, proficiency tests conducted by the EURL-AP are organised under the ISO17043 standard.

The present study report is part of the activity scope of the EURL-AP annual programme.

# 2. Introduction

According to modified Annex VI of Commission Regulation EC/152/2009 [3] official controls for the detection of animal proteins in feed inside the EU have to be performed by light microscopy and/or PCR since June 2013 [4]. Standard Operating Procedures (SOPs) are supporting the implementation of the two methods.

The objective of the present proficiency test was strictly to evaluate within the network of 26 NRLs the analytical performance to detect processed animal proteins (PAPs) in feed by light microscopy and PCR. Participation of the NRLs was mandatory.

In addition, and on proposal of the Commission, invitations to participate to this test were also sent to a limited number of official control labs outside the EU. Non-EU participants were asked to apply also light microscopy and PCR although strict following of Annex VI of Commission Regulation EC/152/2009 and related SOPs was not imposed to them.

# 3. Material and methods

## 3.1. Study organisation

Twenty-six NRLs and six laboratories outside this EU network participated to the study.

Official announcement (Annex 1) of the study was made on 5th September 2025 to all invited participants.

On 24th October 2025, the sample sets were shipped to the participants. On that same day the Excel report forms containing instructions (Annex 2) were also communicated to all participants - downloadable from the EURL-AP intranet for NRLs or sent by email to non-EU participants who have no access to this intranet.

The deadline for the delivery of results was fixed in the announcement and in instructions at the 24th of November 2025.

Within the instructions, some general recommendations were delivered to participants:

- Laboratories participating to the proficiency test were themselves responsible to reach appropriate homogeneity of the sample sub-portions that had to be taken from the whole sample vial for analysis. Precautions to avoid laboratory cross-contamination were also highlighted.

- Results had to be encoded by way of an Excel report form (Annex 2). Participants were asked to carefully read instructions on how to fill in the result form and to testify they did it prior to encoding their results. No other support for communicatingresults was accepted.

- Participants were asked to sign the summarized results sheet that is automatically generated when filling the form and to return it by email to the EURL-AP.  Results were taken into consideration only when both the Excel file and a copy of the summarized results sheet were received by the EURL-AP.

- Participants were notified that results arriving later would not be accepted.

Two participants from the NRL network did not deliver their results on due time and two non-EU participants did not deliver their results at all. Thus, the study presents results from 28 participants. Proficiencies of NRLs and other participants were evaluated separately.

## 3.2. Material

### 3.2.1. Description of the samples

Five different blind test materials were prepared for the study. The composition of the sample set was established considering the following factors:

- Use of feed and feed materials intended to horses and pigs;
- Use of pure fish ingredient commercially available (a tuna fishmeal);
- Use of a compound feed containing dairy product at a level detectable by light microscopy and generating a positive ruminant signal by PCR;
- Use of different terrestrial vertebrate PAPs (from poultry, ovine and porcine origins);
- Use of a pelleted matrix to monitor contamination issues during grinding.

Each participating lab received thus a sample set of five vials, each of about 40 g, to which a unique random number was assigned. Details of the sample set are indicated in Table 1.

**Table 1: Composition of the sample set.**

| Sample | Material | Microscopy (particles) Terrestrial vert. | Terrestrial invert. | Fish | PCR (DNA) Ruminant | Pig | Poultry |
|---|---|---|---|---|---|---|---|
| 1 | Tuna meal + 1 % poultry PAP | + | - | + | - | - | + |
| 2 | Horse feed | - | - | - | n.a. | - | - |
| 3 | Horse feed + 0.05 % ovine PAP | + | n.a. | - | + | - | - |
| 4 | Pig feed (containing milk powder) | + | - | - | + | - | - |
| 5 | Pig feed (containing milk powder) + 0.1 % pig PAP | + | - | - | + | + | - |
| **Total positive results** | | **4** | **0** | **1** | **3** | **1** | **1** |

**(n.a. = not submitted to proficiency assessment, \* = explanations on expected results are described in section 3.4)**

Expected results were internally determined based on the known composition of samples (presence or absence of PAP) and results obtained during the homogeneity study (see 3.5).

### 3.2.2. Materials used in the preparation of samples

Four commercially available feed materials or feed were used as matrices:

- A pure **tuna meal** was used for preparation of sample 1. It had a sediment content of about 25 %. Only particles from fish origin could be detected by microscopy and the only DNA content determined by PCR was fish.

- A first **horse feed**, used to prepare sample 2, was a complement pellet feed. It was composed of linseed, wheat, beet pulp, chicory root, molasse, rapeseed and wheat feed, minerals and vitamins. Its sediment content was limited to 0.2 %. No animal remains could be detected by microscopy. No animal DNA was detected by PCR.

- A second **horse feed,** used in sample 3, was a commercial flake feed. It was composed of barley, pelleted wheat bran, linseed bran, maize flakes, alfalfa, beet molasse, splat soybean oil, minerals and vitamins. Its sediment was of 1.8 %. The feed was free of any animal presence as controlled by microscopy and PCR.

- The last feed, **a piglet feed**, was used for preparing sample 4 and 5. Its composition was unknown aside its declared dairy product and lactose content. Its sediment was of 1 %. Microscopic analyses revealed the presence of lactose crystals. On the exception of ruminant DNA no other presence of animal DNA was detected by PCR.

Adulterant material used:

- A **poultry meal** was used for preparing sample 1. Its sediment was of 23 %. Only poultry DNA was detected by PCR.

- An **ovine PAP** was used to prepare sample 3. Its sediment content was of 28 %. PCR analyses revealed the presence of ruminant (including ovine) DNA. Poultry DNA was also detected in the pure matrix, but at a level too low to be detected in feed adulterated at 0.05%. Microscopic analysis showed a few wool hair presence.

- A **porcine PAP** was also used for sample 5. Its sediment content was of 14.5 %. DNA analysis demonstrated its porcine origin. Trace of ruminant DNA (around the cut-off value) was detected.

### 3.2.3. Description of the mixing procedures

To avoid presence of interfering material, a cleaning of the rooms where samples were handled was performed prior to sample preparation, mixing of materials and filling of vials.

As a rule of best practice, all feed matrices, on the exception of the pelleted horse feed, were ground and conditioned separately. Only after the whole conditioning of the vials, adulterations were realised by direct spiking into the vials. Once the entities were ready and before the homogeneity test was performed, entities were stored at -20°C to prevent mite infestation.

## 3.3. Qualitative analysis

Analyses of qualitative proficiency testing were applied following ISO 13528 [5].

### 3.3.1. Light microscopy

Qualitative analysis concerned the detection of terrestrial vertebrates, terrestrial invertebrates and/or fish material.

Results are expressed by the participants in three formulations according to regulation EU/56/2013 [4] amending regulation EC/152/2009 [3]:

- Positive (= presence of microscopically detectable animal material)
- Negative (= absence of any microscopically detectable animal material)
- Below LOD (= low level presence of microscopically detectable animal material with a risk of false positive result)

Considering the risk of false positive results, all results expressed as below LOD have to be assimilated to negative ones as by definition they cannot be certified as positive *sensu stricto*. This allows an on-off, or binary result analysis.

These binary results were analysed by classical statistics: accuracy, sensitivity and specificity. All those statistics were expressed as fractions.

Accuracy is the fraction of correct positive and negative results; it was calculated by the following equation:

$$\text{Accuracy } AC = \frac{PA + NA}{PA + ND + PD + NA}$$

where *PA* is the number of correct positive results (Positive Agreements), *NA* the number of correct negative results (Negative Agreements), *ND* the number of false negative results (Negative Deviations) and *PD* the number of false positive results (Positive Deviations).

Sensitivity is the ability of classifying positive results as positive, it was calculated as follows:

$$\text{Sensitivity } SE = \frac{PA}{PA + ND}$$

Specificity is the ability of classifying negative results as negative, it was calculated as follows:

$$\text{Specificity } SP = \frac{NA}{PD + NA}$$

The *AC, SE* and *SP* were calculated separately for each laboratory and for each requested parameter (terrestrial vertebrates' material, terrestrial invertebrates' material and of fish material) for the estimation of its proficiency. A consolidated *AC* over the three parameters was used to rank each participant. Finally, a global *AC* was also calculated for each material to estimate the performance of the network.

3.3.2. PCR

Qualitative analysis concerned the detection of ruminant, pig and poultry (chicken-turkey) DNA as prescribed by Annex VI of Commission Regulation (EC) No 152/2009 in its consolidated most recent version complemented by the corresponding binding SOPs.

The participants delivered Ct values (in cycles) to compare to a cut-off value (in cycles). A cut-off value being specific of a PCR test, one must set for the ruminant, the pig and the poultry DNA detection respectively. For the detection of ruminant DNA and poultry DNA, the respective cut-off are set at 15 copies of the target and validated by a quality criterion (the cut-off Ct value must correspond to a number of copies of the target > 9.00 copies). In the case of the detection of pig DNA, the cut-off is set at 5 copies of the target with a quality criterion > 3.00 copies.

For each sample, DNA is extracted from 2 test portions. The results obtained from the 2 test portions must be consistent, in the sense that both Ct values should be close enough to each other and on the same side compared to the cut-off value. A Ct value < cut-off value corresponds to a positive result. Respectively, a Ct value ≥ cut-off value corresponds to a negative result. Results are expressed by the participants in two formulations:

- Present (= presence of targeted DNA detected)
- Absent (= no targeted DNA detected)

As for light microscopy, these binary results were analysed by classical statistics (accuracy, sensitivity and specificity) with the same formulae as presented in 3.3.1.

## 3.4. Performance criteria

Evaluation of the performance and scoring were applied as recommended by ISO 13528 [5].

### 3.4.1. Light microscopy

Considering the sample set composition and the announced parameters (Annex 2), the expected results are indicated in Table 1.

Concerning the presence of terrestrial vertebrates:

- Samples 1, 3, 4 and 5 had to be declared as positive.
- Sample 2 had to be declared negative

Concerning the presence of terrestrial invertebrates:

- All samples had to be declared negative; sample 3 was excluded for proficiency assessment for this parameter.

Concerning the presence of fish:

- Sample 1 had to be declared positive
- Samples 2, 3, 4 and 5 had to be declared negative.

Based on these considerations, the following performance criteria were decided for light microscopy:

- **Excellent** level of global performance: consolidated AC = 1.00 or faultless set of results.

- **Satisfying** level of global performance: consolidated AC ≥ 0.86 without ND for terrestrial vertebrates.

- **Underperforming** level of global performance: consolidated AC < 0.86 OR ≥ 0.86 with one ND for terrestrial vertebrates.

### 3.4.2. PCR

As for light microscopy, the expected results are indicated in Table 1.

- Sample 1 was considered to be positive for the presence of poultry DNA and negative for the other targets.
- Sample 2 had to be declared negative for the presence of pig and poultry DNA.

- Sample 3 and 4 were considered to be declared positive for the presence of ruminant DNA and negative for the other targets..
- Sample 5 was considered to be positive for the presence of ruminant and pig DNA and negative for poultry DNA.

The detection of ruminant DNA for sample 2 was out of the performance assessment.

Concerning the PCR, the performance criteria were decided as:

- **Excellent** level of global performance: global AC = 1.00 with no false result (ND or PD) for the detection of ruminant, pig and poultry DNA.

- **Satisfying** level of global performance: global AC ≥ 0.93 with maximum 1 false result (ND or PD) for the detection of pig and poultry DNA and no deviation for the detection of ruminant DNA.

- **Underperforming** level of global performance: global AC ≥ 0.93 with 1 false result (ND or PD) for the detection of ruminant DNA or global AC < 0.93 with 2 false results (ND or PD) or more.

## 3.5. Homogeneity study

Homogeneity study has been carried out for all materials used.  Table 2 summarizes the results.

**Table 2: Homogeneity study – Results.**

| Sample | Material | Nr of replicates | Light microscopy | | | Nr of replicates | PCR | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Terrestrial vert. | Terrestrial invert. | Fish | | Ruminant | Porcine | Poultry |
| 1 | Tuna meal + 1 % poultry PAP | 10 | + | - | + | 10 | - | - | + |
| 2 | Horse feed | 10 | - | - | - | 10 | - | - | - |
| 3 | Horse feed + 0.05 % ovine PAP | 10 | + | * | - | 10 | + | - | - |
| 4 | Pig feed (containing milk powder) | 10 | + | - | - | 10 | + | - | - |
| 5 | Pig feed (containing milk powder) + 0.1 % pig PAP | 10 | + | - | - | 10 | + | + | - |

**(Legend: ND = not tested, + = systematically detected, - = systematically not detected, * = results not systematically negative)**

The homogeneity was studied by light microscopy on 10 g of sample material for 10 replicates.  Analyses of replicates were performed following EC/152/2009 regulation [3].  According to the proficiency test instructions, all samples were submitted to a double PE/TCE sedimentation. For PCR analysis, a DNA extraction was performed on 2 test portions of 100 mg of sample material for each of the 10 replicates.

**Sample 1** (tuna meal + 1 % poultry PAP) was systematically positive for the presence of both terrestrial vertebrates and fish with respectively observations of bone and fishbone fragments. Muscle fibres and feathers were also detected although feathers were not detected in 2 out of the 10 replicates. No traces of terrestrial invertebrates were detected. PCR analyses detected systematically the presence of poultry DNA whereas ruminant and pig DNA were absent.

**Sample 2** (horse feed) revealed to be totally free from any particle from animal origin. No DNA from ruminant, pig and poultry was detected by PCR analyses.

**Sample 3** (horse feed + 0.05 % ovine PAP) was systematically positive for the presence of terrestrial vertebrates based on the finding of bones. Few animal hairs and muscle fibres were also systematically detected but always at a level never exceeding 5 particles per replicates. No traces of fish could be detected. Regarding the presence of terrestrial invertebrates, a total of 13 insect fragments were identified through the 10 replicates. This resulted in five negative results, four results < LOD and one positive results for this

parameter. For this reason, this parameter was excluded from the proficiency evaluation. Ruminant DNA was systematically detected, while pig and poultry DNA were declared negative.

**Sample 4** (pigfeed containing milk powder) was systematically positive for terrestrial vertebrates due to the presence of abundant small lactose crystals. On the exception of a single muscle fibres, through the series of 10 replicates, no other evidence for particles from animal origin could be detected. PCR analyses detected systematically the presence of ruminant DNA while pig and poultry DNA were absent.

**Sample 5** (pig feed containing milk powder + 0.1 % pig PAP) was positive for the presence of terrestrial vertebrates. Among the terrestrial vertebrates' findings, the presence of bones, muscles and lactose crystals were systematically recorded. No trace of invertebrates or fish could be found. Only DNA from ruminant and pig were detected systematically by PCR analyses. Poultry DNA was not detected.

Results from the homogeneity study allowed declaring the samples as fit for their purpose according to the requested parameters.

## 3.6. Stability of the samples

Internal stability studies performed on similar samples from past studies have demonstrated that such samples were stable over time (years) for both light microscopic and PCR analyses.

# 4. Results

Gross results for microscopy and PCR from all participants are to be found in Annexes 3 and 4 respectively.

## 4.1. Microscopy results

### 4.1.1. Qualitative analyses from the NRLs

#### 4.1.1.1. Results and performance of the network

Table 3 summarizes the results reported by the 24 NRLs for the sample types submitted to microscopic analysis.

The overall results, expressed in terms of global accuracy (AC) reveal the performance of the NRL network for the detection of PAPs from the present test. The percentage of total error accounted for only 7 % of the total responses.

**Table 3: Global results expressed as accuracy (AC) – light microscopy.**

| Sample | Material | n | AC | | |
|--------|----------|---|------------|------------|------------|
| | | | Terr. Vert. | Terr. Inv. | Fish |
| 1 | Tuna meal + 1 % poultry PAP | 24 | 0.875 (3) | 1.000 | 1.000 |
| 2 | Horse feed | 24 | 0.958 (1) | 0.917 (2) | 0.958 (1) |
| 3 | Horse feed + 0.05 % ovine PAP | 24 | 0.958 (1) | n.a | 1.000 |
| 4 | Pig feed (containing milk powder) | 24 | 0.708 (7) | 1.000 | 0.958 (1) |
| 5 | Pig feed (containing milk powder) + 0.1 % pig PAP | 24 | 1.000 | 1.000 | 1.000 |

**Accuracy means sensitivity in case of ND and specificity in case of PD.**
**In brackets the absolute number of ND or PD. (Legend: n = number of results).**

Regarding the detection of terrestrial vertebrates' constituents, some sensitivity issues were noted. These false negative results were spread over samples 1, 3 and 4. They accounted for 69 % of the total errors. The major problem is the lack of detection of dairy component in sample 4. About specificity, only one case of false positive result was found into sample 2.

In absence of insect PAPs, only specificity issues could arise. Two false positive results were recorded for sample 2.

The detection of fish constituents occurred faultless with no sensitivity issues. Only two specificity issues were found, respectively one in sample 2 and a second in sample 4.

Further details on the errors noted in this study are described in next point.

#### 4.1.1.2. Detailed review of results per sample

**Sample 1: Tuna meal + 1 % poultry PAP**

ND for terrestrial vertebrates' particles:

- Lab 11 failed at detecting terrestrial vertebrates' particles

Two laboratories were below the limit of decision:

- Lab 12 reported bones and blood at <LOD level
- Lab 21 reported bones, blood and feathers at <LOD level while commenting the 'not typical' aspect of the bones

The finding of feathers was only reported by 54 % of the NRLs while 25 % of the NRLs did not mention the presence of bones.

All results were correct for the terrestrial invertebrates and fish parameter.

**Sample 2: Horse feed**

PD for terrestrial vertebrates' particles:

- Lab 8 reported the presence of bones

In addition, there was one case of <LOD based on the detection of one feather particle by lab 7.

PD for terrestrial invertebrates' particles:

- Labs 1 and 7 reported the presence of cuticles

PD for fish particles:

- Lab 13 declared the sample as positive without any detailed information on its findings.

**Samples 3: Horse feed + 0.05 % ovine PAP**

ND for terrestrial vertebrates' particles:

- Lab 20 failed at detecting terrestrial vertebrates' particles

Regarding the fish parameter, all results were correct.

The parameter for terrestrial invertebrates was excluded from the proficiency assessment for this sample. Nevertheless, from the results delivered it was interesting to notice that 5 NRLs declared the sample positive for terrestrial invertebrates' presence, 5 others reported findings at <LOD and 14 declared it as negative.

**Sample 4: Pig feed (containing milk powder)**

ND for terrestrial vertebrates' particles:

- Labs 4, 7, 8, 12, 14, 20 and 21 failed at detecting terrestrial vertebrates' particles

From the 17 NRLs that declared sample 4 as positive, 14 referred to the identification of milk, milk powder, milk product or lactose crystals, while 3 didn't mention this presence but instead referring to blood products or muscles.

Regarding the terrestrial invertebrates' parameter, all results were correct.

PD for fish particles:

- Lab 11 declared the sample as positive for fish based on the erroneous muscle finding.

**Sample 5: Pig feed (containing milk powder) + 0.1 % pig PAP**

All results were correct for the three parameters.

There was one case of <LOD for the presence of terrestrial invertebrates based on the detection of one dust mite by lab 7.

Regarding the detection of terrestrial vertebrates, the finding of bones was systematically reported. For this sample, based on the same feed matrix used for sample 4, only 10 NRLs reported the presence of dairy material.

4.1.1.3.   Individual performances of NRLs in qualitative analysis

Individual performance parameters were assessed for each participant by calculating the accuracy, sensitivity and specificity over the blind sample set.  This was calculated separately for each parameter: the detection of terrestrial vertebrates, terrestrial invertebrates and of fish material. Results are to be found in Tables 4, 5 and 6. A ranking of the labs was prepared based on the consolidated accuracy.

**Tables 4 (left) and 5 (right): NRL proficiencies regarding the detection of terrestrial vertebrates and invertebrates' material respectively. Ranking follows AC values for primary key and SE for second key.**

| Terrestrial vert. | | | |
|---|---|---|---|
| lab code | AC | SE | SP |
| 1, 2, 3, 6, 9, 10, 13, 15, 16, 17, 18, 19, 22, 23, 24 and 26 | 1.000 | 1.000 | 1.000 |
| 4, 7, 11 and 14 | 0.800 | 0.750 | 1.000 |
| 8 | 0.600 | 0.750 | 0.000 |
| 12, 20 and 21 | 0.600 | 0.500 | 1.000 |

| Terrestrial inv. | | |
|---|---|---|
| lab code | AC | SP |
| 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 26 | 1.000 | 1.000 |
| 1 and 7 | 0.750 | 0.750 |

**Table 6: NRL proficiencies regarding the detection of fish material. Ranking follows AC values for primary key and SP for second key.**

| Fish | | | |
|---|---|---|---|
| lab code | AC | SE | SP |
| 1, 2, 3, 4, 6, 7, 8, 9, 10, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 26 | 1.000 | 1.000 | 1.000 |
| 11 and 13 | 0.800 | 1.000 | 0.750 |

A general ranking of the NRLs was also performed on a consolidated evaluation including their proficiency in detecting the three parameters through the set of blind samples (Table 7).

**Table 7: General NRL proficiency. Ranking follows AC values as primary key and SE as second key. Lines in black refer to excellent results, lines in blue to satisfying results and lines in red to underperforming results.**

| Consolidated | | | |
|---|---|---|---|
| lab code | AC | SE | SP |
| 2, 3, 6, 9, 10, 15, 16, 17, 18, 19, 22, 23, 24 and 26 | 1.000 | 1.000 | 1.000 |
| 1 and 13 | 0.929 | 1.000 | 0.889 |
| 4 and 14 | 0.929 | 0.800 | 1.000 |
| 7, 8 and 11 | 0.857 | 0.800 | 0.889 |
| 12, 20 and 21 | 0.857 | 0.600 | 1.000 |

From the 24 NRLs, 14 performed excellently (58 %), 2 performed satisfyingly (8 %) and 8 were underperforming (34 %).

In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), the underperforming participants (Labs 4, 7, 8, 11, 12, 14, 20 and 21) are asked to report on the origin of their errors as well as on the actions they will undertake to solve the problems.

4.1.2. <u>Qualitative analyses and individual performances the non-EU participants</u>

Individual performances from the 4 participants outside the EU were assessed exactly as in the previous section (4.1.1.3). A ranking of those labs was prepared as well based on the consolidated accuracy. Results are to be found in Tables 8, 9 and 10.

**Tables 8 (left) and 9 (right): non-EU lab proficiencies regarding the detection of terrestrial vertebrates and invertebrates' material respectively. Ranking follows AC values for primary key and SE for second key.**

| Terrestrial vert. | | | |
|---|---|---|---|
| lab code | AC | SE | SP |
| 27, 28 and 29 | 1.000 | 1.000 | 1.000 |
| 32 | 0.800 | 0.750 | 1.000 |

| Terrestrial inv. | | |
|---|---|---|
| lab code | AC | SP |
| 27, 29 and 32 | 1.000 | 1.000 |
| 28 | 0.250 | 0.250 |

**Table 10: non-EU lab proficiencies regarding the detection of fish material. Ranking follows AC values for primary key and SP for second key.**

| Fish | | | |
|---|---|---|---|
| lab code | AC | SE | SP |
| All labs | 1.000 | 1.000 | 1.000 |

**Sample 1: Tuna meal + 1 % poultry PAP**

All results were correct for the terrestrial vertebrates and fish parameters.

PD for terrestrial invertebrates' particles:

- Lab 28 reported structures looking like tracheal parts of insects

**Sample 2: Horse feed**

All results were correct for the three parameters.

Lab 28 reported the presence of tracheal structures below the limit of decision.

**Samples 3: Horse feed + 0.05 % ovine PAP**

All results were correct for the two parameters.

Regarding the terrestrial invertebrates' parameter, excluded from the proficiency assessment as explained, it has to be noted that lab 29 reported the presence of cuticles and lab 28 reported structures looking like tracheal parts of insects but at a <LOD level.

**Sample 4: Pig feed (containing milk powder)**

ND for terrestrial vertebrates' particles:

- Lab 32 described particles identified as bones and blood but at level <LOD.

Only one participant was able to find the presence of lactose crystals.

PD for terrestrial invertebrates' particles:

- Lab 28 erroneous referred to structures possibly originating from black soldier fly larvae

Regarding the fish parameter, all results were correct.


**Sample 5: Pig feed (containing milk powder) + 0.1 % pig PAP**

All results were correct for the terrestrial vertebrates and fish parameters.

PD for terrestrial invertebrates' particles:

- Lab 28 erroneous referred to structures possibly originating from black soldier fly larvae


A general ranking as for the NRL network was established for non-EU participants (Table 11).

**Table 11: General non-EU lab proficiency. Ranking follows AC values
as primary key and SE as second key. Lines in black refer to excellent
results and lines in red refer to underperforming results.**

| Consolidated lab code | AC | SE | SP |
|---|---|---|---|
| 27 | 1.000 | 1.000 | 1.000 |
| 29 | 1.000 | 1.000 | 1.000 |
| 32 | 0.929 | 0.800 | 1.000 |
| 28 | 0.786 | 1.000 | 0.667 |

Two participants performed excellently, and 2 participants were classified as underperforming according to the applied criteria.


## 4.2. PCR results

4.2.1. Qualitative analyses from the NRLs

4.2.1.1. On the respect of the instructions

The NRLs generally comply with SOPs. However, some labs do not use one of the mastermixes recommended by the EURL-AP: Lab 2 uses the TaqMan™ Universal PCR Master Mix (ThermoFisher Scientific) for the detection of ruminant DNA while it uses the Brilliant II QPCR Low ROX Master Mix (Agilent) for the detection of pig and poultry DNA. Lab 14 encoded the Maxima Probe qPCR Master Mix (2x) from ThermoFisher Scientific. Labs 15 and 19 performed the reactions with a mastermix supplied by BIOSENSE without further information. Lab 20 used a mastermix provided by LaCar. This often had no impact on the results but Labs 14 and 20 recorded deviations leading to poor performances.

4.2.1.2. Overview of results and global performance of the network

Table 12 summarizes the results provided by 24 NRLs for the five samples submitted to qualitative PCR analysis.

**Table 12: Global results expressed as accuracy (AC) – PCR.**

| Sample | Material | n | AC | | |
|---|---|---|---|---|---|
| | | | Ruminant | Pig | Poultry |
| 1 | Tuna meal + 1 % poultry PAP | 24 | 0.958 (1) | 1.000 | 1.000 |
| 2 | Horse feed | 24 | n.a. | 0.958 (1) | 1.000 |
| 3 | Horse feed + 0.05 % ovine PAP | 24 | 0.958 (1) | 1.000 | 1.000 |
| 4 | Pig feed (containing milk powder) | 24 | 1.000 | 1.000 | 1.000 |
| 5 | Pig feed (containing milk powder) + 0.1 % pig PAP | 24 | 1.000 | 0.958 (1) | 1.000 |

**Accuracy means sensitivity in case of ND and specificity in case of PD.**
**In brackets the absolute number of false results. (Legend: n = number of results)**
**n.a. = not submitted to proficiency assessment.**

On the overall results, only 4 deviations (1.2 % out of the 336 results) were recorded. With the poultry target, the results are perfect. The rate of false results obtained with the ruminant and the pig assay are 2.1 % and 1.7 % respectively which is fully acceptable.

**Sample 1: Tuna meal + 1 % poultry PAP**

The PCR results expected were the presence of poultry DNA only. All the results for the detection of porcine and poultry DNA were correct. For the detection of ruminant DNA, one positive deviation was recorded by Lab 4.

**Sample 2: Horse feed**

The PCR results expected were the absence pig and poultry DNA. The detection of ruminant DNA was kept out of the proficiency assessment. One positive deviation for the detection of pig DNA was recorded by Lab 14. All the remaining results were correct.

**Sample 3: Horse feed + 0.05 % sheep PAP**

The PCR results expected were the presence of ruminant DNA only. Lab 20 did not detect the presence of ruminant DNA. All the results for the detection of porcine and poultry DNA were correct.

**Sample 4: Pig feed (containing milk powder)**

The PCR results expected were the absence pig and poultry DNA whereas ruminant DNA was expected to be present. All the results recorded were correct.

**Sample 5: Pig feed (containing milk powder) + 0.1 % pig PAP**

The PCR results expected were the presence of ruminant and pig DNA. All the results for the detection of ruminant and poultry DNA were correct but one negative deviation for the detection of pig DNA was recorded (Lab 14).

4.2.1.3. Individual performances of NRLs in qualitative analysis

Individual performances were assessed for each participant by calculating the accuracy, sensitivity and specificity over the samples. A ranking of the labs was prepared based on the accuracy. Results are to be found in Table 13 that summarizes the results obtained by the participants.

**Table 13: NRL proficiencies regarding the detection of ruminant, pig and poultry DNA. Ranking follows AC values. Cells in black refers to excellent NRLs. Cells in blue refers to satisfying NRLs. Cells in red refer to underperforming NRLs.**

| Lab code | AC | SE | SP |
|---|---|---|---|
| 1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 21, 22, 23, 24 and 26 | 1.000 | 1.000 | 1.000 |
| 4 | 0.929 | 1.000 | 0.889 |
| 20 | 0.929 | 0.800 | 1.000 |
| 14 | 0.857 | 0.800 | 0.889 |

Excellent performances were recorded for 21 labs out of 24 NRLs (88 % of the NRLs) having no false result.

Three labs (Lab 4, 14 and 20) are under-performing. Lab 4 and 20 recorded one deviation with the ruminant test. Lab 14 obtained two deviations (one positive and the other negative) with the pig assay.

4.2.1.4. Cut-off quality control

A quality control for the number of copies of the target reached with the Ct value of the cut-off, was developed to minimize the risk of false positive result. A minimum of 9.00 copies at the cut-off is required for the ruminant and the poultry PCR tests whereas it is 3.00 copies at the cut-off for the pig PCR test. Indeed, depending on the variability of the lab (PCR platform + operator), the cut-off value can correspond to a too low number of copies.

Except Lab 1, all other participants reached the minimum criterion of 9.00 copies for the ruminant cut-off. The range of copies at the cut-off goes from 8.98 copies to 13.97 copies. The cut-off, in cycles, is comprised between 31.84 cycles and 37.51 cycles. The percentage of the labs with a cut-off corresponding to a number of copies > 10 for this proficiency test was 75.0 %.

For the pig cut-off, all participants reached the minimum criterion set at 3.00 copies. The range of copies at the cut-off goes from 3.06 copies to 4.77 copies. The cut-off, in cycles, is comprised between 35.02 cycles and 42.12 cycles. The percentage of the labs with a cut-off corresponding to a number of copies > 3.50 for this proficiency test was 75.0 %.

Considering the poultry cut-off, all NRLs reached the minimum criterion of 9.00 copies. The range of copies at the cut-off goes from 9.02 copies to 13.74 copies. The cut-off, in cycles, is comprised between 34.83 cycles and 45.00 cycles.

It should be noted that Lab 1 has not updated any of its thresholds since PT 2024, even though the minimum quality criterion for the ruminant threshold was not met (8.98 copies). Laboratories 10 and 15 also use 'old' thresholds set two or three years ago, with the risk of obtaining erroneous results in case of deviation of their PCR platform.

4.2.2. Qualitative analyses from the non-EU participants

4.2.2.1. Individual performances

Individual performances were assessed for three non-EU participants who reported PCR results by calculating the accuracy, sensitivity and specificity over the samples. Their results are to be found in Table 14.

**Table 14: Non-EU participant proficiencies regarding the detection of ruminant, pig and poultry DNA. Ranking follows AC values. Cells in black refers to excellent labs. Cells in blue refers to satisfying labs. Cells in red refers to underperforming labs.**

| Lab code | AC | SE | SP |
|---|---|---|---|
| 29 | 1.000 | 1.000 | 1.000 |
| 28 | 0.929 | 1.000 | 0.889 |
| 27 | 0.929 | 0.800 | 1.000 |
| 32 | 0.929 | 0.800 | 1.000 |

Labs 29 obtained excellent results (no deviation).

Labs 27, 28 and 32 were assessed as satisfactory. These participants reported one deviation for the detection of pig DNA. Lab 27 and 32 obtained one negative deviation (with sample 5) and Lab 28 had one positive deviation with sample 3.

4.2.2.2. Assessment of the cut-off values

Labs 28 and 29 have cut-off values that comply with the minimum criteria (9 copies for the ruminant and the poultry PCR tests; 3 copies for the pig PCR test) set by the EURL-AP. Except for the ruminant DNA detection, Lab 27 used home-made methods without cut-off values. Lab 32 also used home-made PCR methods.

# 5. Discussion and conclusions

The overall performance for the microscopic detection of PAPs of the NRL network for the present study was satisfying in comparison with former studies. The total error rate was of 7 %. The number of excellent and satisfactory scores obtained within the network of NRLs reached respectively 58 % (73 % in 2024 [6] and 54 % in 2023 [7]) and 2 % (23 % in 2024 [6] and 38 % in 2023 [7]). The rate of underperforming NRLs for the present study was increased to 34 % (4 % in 2024 [6] and 8 % in 2023 [7]). This increase of underperforming participant deserves some comments.

Most of the errors were related to sensitivity issues in detecting terrestrial vertebrate material, accounting for 69 % of the total errors. Among these shortcomings, the detection of milk-derived materials remains particularly challenging, as illustrated by the difficulty in identifying lactose crystals. Although the homogeneity study showed an abundance of small crystals, only 14 NRLs succeeded in detecting their presence. It should also be noted that, for this sample, some correct positive declarations for terrestrial vertebrate material were made based on the erroneous identification of blood products or muscle tissues. This further obscure the true, and likely even lower, detection capability for dairy products than what was observed. Consequently, targeted efforts are needed within the network to strengthen detection skills for these specific animal-derived components.

The second main source of error was the difficulty in identifying poultry PAP remains within the fish meal. This corresponds to the well-known and old-dating masking effect described in previous studies [for review, 8], where the abundance of fish fragments in the sediment conceals terrestrial bones—in this case, avian bones—even at high adulteration levels (1 %). However, in the present study, since the proportion of sediment originating from the matrix was similar to that of the adulterant (25 % and 23 %, respectively), the masking effect cannot be attributed to a dilution phenomenon. Instead, it appears to result from a systematic tendency to overlook poultry bones in the slides prepared from the recovered sediments.

Concerning results from the non-EU participants, the detection of terrestrial vertebrates and fish material was not problematic. The only recurrent problem among these participants was a specificity issue for the erroneous finding of insect fragments in absence of any insect PAP material in the sample set. This difficulty was link to only one participating laboratory.

Performances of the NRL network were assessed using the three PCR tests (ruminant, pig and poultry) validated and implemented in the network. All samples had to be analysed by PCR regardless of the results of light microscopy. Consequently, the PCR results accurately reflect the actual performance of participants using these methods.

Four deviations out of 364 PCR results, representing 1.1 % of the results, were observed. All deviations were concentrated in a very limited number of labs. This means that 21 NRLs perfectly managed the analyses of the set of samples while three NRLs were declared underperforming. Two NRLs were recorded underperforming due to a strict performance criterion (no deviation allowed) concerning the detection of ruminant DNA. The third NRL sent back two deviations. Results of two remaining NRLs were kept out of the report by organisers due to a reporting after the deadline.

Four non-EU laboratories also analysed the samples. One of them performed excellently while the three others were satisfying.

# Acknowledgment

# References

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# Annex 1

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## Announcement of the EURL-AP proficiency test 2025/01 for the determination of Processed Animal Proteins (PAPs) in feed

### Introduction

The use of processed animal by-products as ingredient for animal feedingstuffs within the European Union is regulated by the TSE Regulation (Regulation EC N°999/2001), as amended. In particular, Article 7 imposes a prohibition to use processed animal proteins in the feeding of farmed animals (extended feed ban).

Commission implementing Regulation (EU) No 2022/893 amending Annex VI of Regulation (EC) No 152/2009, imposes the methods of analysis for the determination of constituents of animal origin for the official control of feed.

### Objectives

The objective of the present proficiency test is to assess the performance of the NRLs to detect the presence of PAPs in feed by the reference methods using light microscopy and PCR as stated in Regulation EC 152/2009 as amended by Commission implementing Regulation (EU) No 2022/893 and related SOPs.

### The organizer team

The test will be coordinated by the European Union Reference Laboratory for Animal Proteins in feedingstuffs (EURL-AP).

### Test material

Samples containing typical compound feed fortified with processed animal proteins (PAPs) will be prepared. The EURL-AP will endorse the homogeneity of the samples. Nevertheless, each laboratory participating to the test is sole responsible to reach appropriate homogeneity for the sample sub-portions taken for analysis.

Each participant will receive a maximum of 5 samples, each of about 40g. Each sample, identified by a unique numerical code, shall be analysed both by light microscopy and PCR.

### General outline of the exercise

- The light microscopic and PCR methods to use are described in Annex VI of Commission Regulation EC 152/2009 and related SOPs.
- Parameters that will be assessed are: terrestrial vertebrates, terrestrial invertebrates and fish presence/absence, DNA presence/absence from ruminants, pig and poultry.
- The EURL-AP will provide participants with an Excel file for reporting the results of the proficiency test analyses.
- Each participating laboratory will be assigned a unique code and only the organizer of the study knows the key to this code. After completing the test each laboratory will get a report including its results and lab code. A final report of the study will be published with anonymised results thus the strict confidentiality of the participants is guaranteed by the organizer. Nevertheless, for underperforming NRLs only, and according to DG Santé protocol WS07/06 related to the management of underperformances, the organizer may be in the situation of informing the Commission of the underperformance or the absence of collaboration.
- The participation in this proficiency study is mandatory and free of charge for national reference laboratories within Member States of the European Union.

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## Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : **5 September 2025**
- Sending of the sample boxes and communication of the instructions : **24 October 2025**

> *By default, samples will be sent to the <u>NRL microscopy contact person</u> referred on the intranet. You are asked to check if this person is still your contact and to inform the organizer from any change.*

- Deadline for returning of results to the organizer : **24 November 2025**

## Further information

- Refer to the address and coordinates mentioned in the heading,

  or

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# Annex 2

## Proficiency Test Microscopy-PCR 2025/01

**Laboratory identification**

Laboratory code : 1

Responsibility agreement : No

*"Yes" means you have read carefully the "Instructions" work sheet
and its accurate application through the present study.*

**Report**

| Lab code | 1 | 1 | 1 | 1 | 1 |
|---|---|---|---|---|---|
| Sample rank | 1st | 2nd | 3th | 4th | 5th |
| **Sample N°** | | | | | |

### Light microscopy

**Light microscopy analyses**

**Terrestrial vertebrates particles**

details of particles
*Only to fill in if in the cell above "present" or "< LOD" is chosen.*

**Terrestrial invertebrates particles**

details of particles
*Only to fill in if in the cell above "present" or "< LOD" is chosen.*

**Fish particles**

details of particles
*Only to fill in if in the cell above "present" or "< LOD" is chosen.*

Number of determinations

Free comment
*Example : presence of unusual fragments,…*

### PCR

**PCR analyses**

| | Ruminant | Poultry | Pig |
|---|---|---|---|
| Cut-off at 15 *(5 for pig)* copies of the PCR platform used (in cycles) | | | |
| Copy number at the cut-off of the PCR platform used (in copies) | | | |
| Maxter mix used | | | |

**Sample N°**

**Ruminant DNA**

Dilution 1 (e.g. 1 fold)
Ct value replicate 1
Ct value replicate 2

Dilution 2 (e.g. 10 fold)
Ct value replicate 1
Ct value replicate 2

**Poultry DNA**

Dilution 1 (e.g. 1 fold)
Ct value replicate 1
Ct value replicate 2

Dilution 2 (e.g. 10 fold)
Ct value replicate 1
Ct value replicate 2

**Pig DNA**

Dilution 1 (e.g. 1 fold)
Ct value replicate 1
Ct value replicate 2

Dilution 2 (e.g. 10 fold)
Ct value replicate 1
Ct value replicate 2

Free comment
*Example : PCR inhibition,…*

# Annex 3

## Gross results of participants for microscopy (in numerical order of lab ID)

**Laboratory identification code :1**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 2 | 202 | Absent | | Present | cuticula | Absent | | 1 |
| 3 | 259 | Present | bones | Absent | | Absent | | 1 |
| 5 | 661 | Present | bones, milk | Absent | | Absent | | 1 |
| 4 | 802 | Present | milk | Absent | | Absent | | 1 |
| 1 | 973 | Present | bones, feathers | Absent | | Present | bones, gills | 1 |

**Laboratory identification code :2**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 5 | 157 | Present | terrestrial bones, muscle, lactose crystals | Absent | N/A | Absent | N/A | 2 |
| 1 | 451 | Present | terrestrial bones, muscle | Absent | N/A | Present | fish bones, gill, fish tooth | 2 |
| 3 | 637 | Present | terrestrial bones, hair | < LOD | insect appendages | Absent | N/A | 2 |
| 2 | 886 | Absent | N/A | Absent | N/A | Absent | N/A | 1 |
| 4 | 946 | Present | lactose crystals | Absent | N/A | Absent | N/A | 2 |

*Samples 157 & 946 contained unidentified globular structures similar to milk globules but non-conclusive. Sample 451 had a positive blue colour-change reaction to TMB+H202 stain suggesting the potential presence of blood products, however there was no development of air bubbles. In sample 637 we noticed two whole insect appendages below the limit of decision.*

**Laboratory identification code :3**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 2 | 22 | Absent | | Absent | | Absent | | 1 |
| 1 | 55 | Present | feather meal, bones | Absent | | Present | fish bones, gills, muscle fibers | 2 |
| 4 | 226 | Present | milk products | Absent | | Absent | | 1 |
| 3 | 277 | Present | bones | Absent | | Absent | | 1 |
| 5 | 895 | Present | bones, milk products | Absent | | Absent | | 1 |

*An insect head was detected in sample 277, despite that double sedimentation had not been performed.*

**Laboratory identification code :4**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 5 | 31 | Present | Bones | Absent | | Absent | | 2 |
| 4 | 118 | Absent | | Absent | | Absent | | 2 |
| 2 | 220 | Absent | | Absent | | Absent | | 2 |
| 3 | 331 | Present | Bones | Absent | | Absent | | 2 |
| 1 | 559 | Present | Bones, feathers | Absent | | Present | Bones, gills, muscle fibers | 2 |

*For 118 and 220, we marked 2 determination because the double sedimentation was performed to look for terrestrial invertebrates, thus making it 2 determinations for these samples*

**Laboratory identification code :6**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 1 | 361 | Present | feather | Absent | | Present | fishbones, scales, muscle fibers | 1 |
| 2 | 382 | Absent | | Absent | | Absent | | 1 |
| 4 | 478 | Present | blood products | Absent | | Absent | | 1 |
| 3 | 619 | Present | bones | Absent | | Absent | | 1 |
| 5 | 913 | Present | bones, blood products | Absent | | Absent | | 1 |

Laboratory identification code : **7**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 1 | 163 | Present | feather particles | Absent | | Present | fish bones, gills | 2 |
| 3 | 295 | Present | bone fragments | Present | cuticules | Absent | | 2 |
| 5 | 319 | Present | bone | < LOD | 1 dust mite | Absent | | 2 |
| 2 | 400 | < LOD | 1 feather particle | Present | cuticules | Absent | | 2 |
| 4 | 784 | Absent | | Absent | | Absent | | 1 |

*We found a dust mite in sample 319.*

*1 feather particle in sample 400 was found in first determination. Therefore, a second determination was performed.*

Laboratory identification code : **8**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 4 | 28 | Absent | | Absent | | Absent | | 1 |
| 3 | 79 | Present | bones, muscle fibres it can't be exluded muscle fibres found only derive from terr. vertebrates | Absent | | Absent | | 1 |
| 5 | 535 | Present | bones | Absent | | Absent | | 1 |
| 2 | 724 | Present | bones | Absent | | Absent | | 1 |
| 1 | 739 | Present | blood products (plasma) | Absent | | Present | fishbones, scales, muscle fibres,... it can't be exluded muscle fibres found only derive from fish | 1 |

*Sample 724 showed a mite investation*

Laboratory identification code : **9**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 1 | 325 | Present | Feathers, muscles | Absent | | Present | bones, muscles | 1 |
| 4 | 442 | Present | blood products | Absent | | Absent | | 1 |
| 2 | 472 | Absent | | Absent | | Absent | | 1 |
| 5 | 607 | Present | bones | Absent | | Absent | | 1 |
| 3 | 835 | Present | bones | Absent | | Absent | | 1 |

Laboratory identification code : **10**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 4 | 46 | Present | lactose crystals | Absent | | Absent | | 2 |
| 5 | 139 | Present | lactose crystals, bones | Absent | | Absent | | 2 |
| 3 | 241 | Present | bones | Present | leg,cuticule, muscle | Absent | | 2 |
| 1 | 703 | Present | bones | Absent | | Present | fish bones | 2 |
| 2 | 922 | Absent | | Absent | | Absent | | 1 |

Laboratory identification code : **11**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 2 | 256 | Absent | | Absent | | Absent | | 1 |
| 4 | 334 | Present | muscle | Absent | | Present | muscle | 1 |
| 5 | 553 | Present | bones muscle | Absent | | Absent | | 1 |
| 3 | 763 | Present | bones feathers | Present | cuticule fragments | Absent | | 2 |
| 1 | 829 | Absent | | Absent | | Present | fishbones gills muscles blood teeth cartilage | 1 |

*334 only muscle fibres detected so we have been unable to determine whether it is terrestrial vertebrate or fish.*

## Laboratory identification code : **12**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 1 | 343 | < LOD | Bones, blood | Absent | | Present | Fish bones, cartilages, gills, teeth, muscle fibers, blood | 2 |
| 3 | 385 | Present | Bones, few muscle fibers | Absent | | Absent | | 1 |
| 5 | 643 | Present | Bones, cartilages and few muscle fibers | Absent | | Absent | | 1 |
| 4 | 658 | Absent | | Absent | | Absent | | 2 |
| 2 | 958 | Absent | | Absent | | Absent | | 2 |

*Sample 958: A single insect structure was identified. As no other invertebrate structures were found, it was decided to give a negative result for invertebrates.*

## Laboratory identification code : **13**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 4 | 388 | Present | bone fragments, milk | Absent | | Absent | | 2 |
| 5 | 409 | Present | bone fragments, muscle fibers | Absent | | Absent | | 2 |
| 2 | 688 | Absent | | Absent | | Present | | 1 |
| 3 | 745 | Present | bone fragments, muscle fibers | Absent | | Absent | | 2 |
| 1 | 919 | Present | feathers | Absent | | Present | bone fragments, muscle fibers, gills | 2 |

*1 single TI fragment was detected in sample 409*

## Laboratory identification code : **14**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 5 | 67 | Present | bones | Absent | | Absent | | 2 |
| 2 | 76 | Absent | | Absent | | Absent | | 2 |
| 1 | 289 | Present | bones | Absent | | Present | bones, muscle | 2 |
| 4 | 640 | Absent | | Absent | | Absent | | 2 |
| 3 | 817 | Present | bones | Absent | | Absent | | 2 |

## Laboratory identification code : **15**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 3 | 349 | Present | Bones | < LOD | Cuticule | Absent | | 2 |
| 1 | 397 | Present | Bones | Absent | | Present | Bones | 1 |
| 2 | 454 | Absent | | Absent | | Absent | | 2 |
| 4 | 550 | Present | Milk products | Absent | | Absent | | 1 |
| 5 | 589 | Present | Bones, milk products | Absent | | Absent | | 1 |

## Laboratory identification code : **16**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 3 | 7 | Present | bones, muscles | Absent | | Absent | | 1 |
| 2 | 328 | Absent | | Absent | | Absent | | 1 |
| 1 | 379 | Present | bones, muscles, cartilages | Absent | | Present | fishbones, gills, muscles, cartilages | 1 |
| 5 | 499 | Present | bones, muscles, lactose crystals | Absent | | Absent | | 1 |
| 4 | 856 | Present | muscles, lactose crystals | Absent | | Absent | | 1 |

## Laboratory identification code : **17**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 2 | 310 | Absent | | Absent | | Absent | | 1 |
| 4 | 514 | Present | milk | Absent | | Absent | | 2 |
| 5 | 625 | Present | bones, muscles | Absent | | Absent | | 2 |
| 1 | 865 | Present | feathers | Absent | | Present | bones, scales | 2 |
| 3 | 979 | Present | bones, hair | < LOD | Cuticule | Absent | | 2 |

## Laboratory identification code : **18**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 4 | 100 | Present | milk product | Absent | | Absent | | 1 |
| 5 | 517 | Present | bones, muscles, cartilage, milk product | Absent | | Absent | | 1 |
| 1 | 613 | Present | bones, feathers, blood | Absent | | Present | bones, cartilage, tooth, muscles | 1 |
| 3 | 727 | Present | bones | Absent | | Absent | | 1 |
| 2 | 814 | Absent | | Absent | | Absent | | 1 |

*In the sample 100 we saw some fragments that resembled from horn or a hoof/cloven hoof material. It might not be the case, which is why we didn't include it in the description above.*


## Laboratory identification code : **19**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 3 | 133 | Present | bones | < LOD | | Absent | | 1 |
| 5 | 211 | Present | bones, Milk product | Absent | | Absent | | 1 |
| 1 | 415 | Present | bones | Absent | | Present | | 1 |
| 4 | 892 | Present | milk product | Absent | | Absent | | 1 |
| 2 | 940 | Absent | | Absent | | Absent | | 1 |


## Laboratory identification code : **20**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 3 | 61 | Absent | | Present | parts of insects | Absent | | 2 |
| 2 | 544 | Absent | | Absent | | Absent | | 2 |
| 5 | 715 | Present | bones and muscles | Absent | | Absent | | 2 |
| 1 | 721 | Present | bones, muscles and cartilage | Absent | | Present | bones, cartilage, teaths, gills and muscles | 1 |
| 4 | 964 | Absent | | Absent | | Absent | | 2 |

*Sample 964 has been analyzed for milk, plasma, blood and blod products, but none of it has been detected. A low level of positive identification of blood has been seen, but not sufficient to categorize it as positive. In that sample a single hair was found. In addition an unknown structure was found in sample 964, but it could not be identified. Images of the hair and the not detected structure will be attached to the e-mail.*


## Laboratory identification code : **21**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 1 | 73 | < LOD | >LOD bone, >LOD Feather, >LOD blood | Absent | | Present | bone, meat, cartilage, gills, tooth, scales | 2 |
| 5 | 175 | Present | bone, cartilate, >LOD meat | Absent | | Absent | | 1 |
| 3 | 475 | Present | uncharacteristic bones | < LOD | Cuticula with sensilla. Could be from natrual source. | Absent | | 2 |
| 2 | 580 | Absent | | Absent | | Absent | | 1 |
| 4 | 622 | Absent | | Absent | | Absent | | 1 |

*Sample 73 was very strange in structure. Terrestrial bones present in the samples was not typical.*


## Laboratory identification code : **22**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 2 | 40 | Absent | | Absent | | Absent | | 1 |
| 5 | 247 | Present | Bones, milk powder derivatives (lactose) | Absent | | Absent | | 1 |
| 3 | 421 | Present | Bones, muscle fibers | Absent | | Absent | | 1 |
| 1 | 469 | Present | Bones, muscle fibers, blood products (plasma) | Absent | | Present | scales, fish bones, cartilages, muscle fibers, gills | 1 |
| 4 | 694 | Present | Milk powder derivatives (lactose) | Absent | | Absent | | 1 |

*Sample 469 : The TMB test reveals the presence of blue coloration without bubbles. The other samples are TMB-.*

## Laboratory identification code : 23

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 1 | 91 | Present | bones, feathers and muscles, blood | Absent | | Present | muscles, gills, fishbones | 2 |
| 4 | 352 | Present | milk | Absent | | Absent | | 2 |
| 2 | 562 | Absent | | Absent | | Absent | | 1 |
| 3 | 583 | Present | bones | Absent | | Absent | | 2 |
| 5 | 967 | Present | bones | Absent | | Absent | | 2 |

## Laboratory identification code : 24

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 2 | 274 | Absent | | Absent | | Absent | | 1 |
| 3 | 313 | Present | Bones in the sediment | Present | Cuticles in the flotate | Absent | | 1 |
| 5 | 445 | Present | Bones in the sediment and milk in the flotate | Absent | | Absent | | 1 |
| 4 | 496 | Present | Milk in the flotate | Absent | | Absent | | 1 |
| 1 | 649 | Present | Bones in the sediment and futhers in the flotate | Absent | | Present | Bones, gills, scales, muscle fibers | 1 |

*Sample 496 was suspect for presence of plasma in the flotate*

## Laboratory identification code : 26

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 5 | 301 | Present | Bones | Absent | | Absent | | 2 |
| 3 | 457 | Present | Bones | Absent | | Absent | | 2 |
| 1 | 577 | Present | Bones, feathers, muscle fibers | Absent | | Present | Fishbones, muscle fibers, scales, gills | 2 |
| 4 | 676 | Present | Milk | Absent | | Absent | | 2 |
| 2 | 706 | Absent | | Absent | | Absent | | 2 |

## Laboratory identification code : 27

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 3 | 25 | Present | Bone | Absent | | Absent | | 1 |
| 2 | 166 | Absent | | Absent | | Absent | | 2 |
| 4 | 748 | Present | Bone and muscle fibres | Absent | | Absent | | 2 |
| 1 | 775 | Present | Bone and muscle fibres | Absent | | Present | Bone, muscle fibres, gill, cartilage | 1 |
| 5 | 985 | Present | Bone | Absent | | Absent | | 1 |

## Laboratory identification code : 28

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 3 | 97 | Present | Not extremely many positive, but absolutely more than 5. | < LOD | One structure looks like a leg with hairs and some "shiny" tracheal-looking structures. | Absent | | 1 |
| 1 | 127 | Present | Looks very much like terrestrial particles. | Present | Bright yellow/ochre structures. Yellow mealworm? Some structures look like tracheal parts. | Present | Lots of bones, scales and gills. Mostly just fish particles. | 1 |
| 4 | 586 | Present | Mostly just black and grey particles and some pink. Looks like terrestrial. | Present | Quite many structures with the tiny holes (Black soldier fly larvae?). | Absent | | 1 |
| 5 | 751 | Present | Lots of black particles and shiny minerals. Many terrestrial. | Present | Lots of "Black soldier fly larvae" structures. | Absent | | 1 |
| 2 | 850 | Absent | | < LOD | Tracheal structures. | Absent | | 1 |

*Sample 850 had almost no sediment. No blood or feathers were detected in any of the samples.*

**Laboratory identification code : 29**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 5 | 49 | Present | milk (lactose crystals), bones, muscles | Absent | | Absent | | 2 |
| 4 | 172 | Present | milk (lactose crystals) | Absent | | Absent | | 2 |
| 3 | 205 | Present | bones | Present | cuticule | Absent | | 2 |
| 1 | 433 | Present | bones | Absent | | Present | bones, gills, muscles | 2 |
| 2 | 436 | Absent | | Absent | | Absent | | 2 |

**Laboratory identification code : 32**

| Sample type | Sample N° | Terrestrial vert. part. | Details of terrestrial vert. part. | Terrestrial invert. part. | Details of terrestrial invert. part. | Fish part. | Details of fish part. | Number of determinations |
|---|---|---|---|---|---|---|---|---|
| 2 | 148 | Absent | | Absent | | Absent | | 2 |
| 4 | 208 | < LOD | Bones and Blood | Absent | | Absent | | 2 |
| 1 | 595 | Present | Bones and muscle | Absent | | Present | Bones and muscle | 2 |
| 5 | 679 | Present | Bones and Blood | Absent | | Absent | | 2 |
| 3 | 691 | Present | Bones and Blood | Absent | | Absent | | 2 |

*The presence of blood was detected in sample 148*

# Annex 4

Gross results of participants for PCR (in numerical order of lab ID)

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Laboratory identification code :      **1**

|  | Ruminant | Poultry | Pig |  |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 32,93 | 39,85 | 42,12 | cycles |
| Copy number at the cut-off : | 8,98 | 10,42 | 3,55 | copies |
| Master mix used : | qPCR Mastermix plus w/o UNG, Eurogentec | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 2 | 202 | Absent | Absent | Absent | |
| 3 | 259 | Present | Absent | Absent | |
| 5 | 661 | Present | Absent | Present | |
| 4 | 802 | Present | Absent | Absent | |
| 1 | 973 | Absent | Absent | Absent | |

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Laboratory identification code :      **2**

|  | Ruminant | Poultry | Pig |  |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 36,42 | 36,85 | 37,74 | cycles |
| Copy number at the cut-off : | 10,41 | 10,39 | 3,87 | copies |
| Master mix used : | Ruminant : Taqman Universal PCR Master Mix (Thermofisher, product code 4318157); | | | |
|  | Poultry and Pig : Brilliant II QPCR Low ROX Master Mix (Agilent, product code 600806) | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 5 | 157 | Present | Absent | Present | N/A |
| 1 | 451 | Absent | Present | Absent | N/A |
| 3 | 637 | Present | Absent | Absent | N/A |
| 2 | 886 | Absent | Absent | Absent | N/A |
| 4 | 946 | Present | Absent | Absent | N/A |

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Laboratory identification code :      **3**

|  | Ruminant | Poultry | Pig |  |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 36,63 | 38,74 | 39,40 | cycles |
| Copy number at the cut-off : | 10,30 | 9,79 | 3,63 | copies |
| Master mix used : | Brilliant II QPCR Low Rox Master Mix | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 2 | 22 | Absent | Absent | Absent | |
| 1 | 55 | Absent | Present | Absent | |
| 4 | 226 | Present | Absent | Absent | |
| 3 | 277 | Present | Absent | Absent | |
| 5 | 895 | Present | Absent | Present | |

Laboratory identification code : **4**

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 32,19 | 34,83 | 36,56 | cycles |
| Copy number at the cut-off : | 10,87 | 10,73 | 3,87 | copies |
| Master mix used : | qPCR MasterMix No Rox, RT-QP2X-03+NR (eurogentec) | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 5 | **31** | Present | Absent | Present | no PCR inhibition |
| 4 | **118** | Present | Absent | Absent | no PCR inhibition |
| 2 | **220** | Present | Absent | Absent | no PCR inhibition |
| 3 | **331** | Present | Absent | Absent | no PCR inhibition |
| 1 | **559** | Present | Present | Absent | no PCR inhibition |

Laboratory identification code : **5**

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 34,48 | 36,72 | 36,40 | cycles |
| Copy number at the cut-off : | 10,31 | 11,88 | 3,75 | copies |
| Master mix used : | Agilent Brilliant II low ROX, LOT:6659492 | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 4 | **10** | Present | Absent | Absent | no inhibition |
| 5 | **121** | Present | Absent | Present | no inhibition |
| 3 | **601** | Present | Absent | Absent | no inhibition |
| 1 | **667** | Absent | Present | Absent | no inhibition |
| 2 | **760** | Absent | Absent | Absent | no inhibition |

Laboratory identification code : **6**

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 36,43 | 44,15 | 38,95 | cycles |
| Copy number at the cut-off : | 10,29 | 9,31 | 3,08 | copies |
| Master mix used : | qPCR MasterMix" reference number RT-QP2X-03 | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 1 | **361** | Absent | Present | Absent | |
| 2 | **382** | Absent | Absent | Absent | |
| 4 | **478** | Present | Absent | Absent | |
| 3 | **619** | Present | Absent | Absent | |
| 5 | **913** | Present | Absent | Present | |

Laboratory identification code : **7**

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 35,70 | 36,00 | 37,10 | cycles |
| Copy number at the cut-off : | 11,65 | 10,96 | 4,14 | copies |
| Master mix used : | Takyon Low Rox Probe MasterMix dTTP Blue (18S, ruminant), | | | |
| | Eurogentec RT-QP2X-03WOU+ (No UNG) (pig, poultry) | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 1 | **163** | Absent | Present | Absent | 18S qPCR: no inhibition observed. |
| 3 | **295** | Present | Absent | Absent | 18S qPCR: no inhibition observed. |
| 5 | **319** | Present | Absent | Present | 18S qPCR: no inhibition observed. |
| 2 | **400** | Present | Absent | Absent | 18S qPCR: no inhibition observed. The positive ruminant signal might be due to a contamination from other highly positive samples. 163 and 400 were therefore repeated. 400 stayed positive. |
| 4 | **784** | Present | Absent | Absent | 18S qPCR: no inhibition observed. |

## Laboratory identification code : **8**

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 35,80 | 39,70 | 41,06 | cycles |
| Copy number at the cut-off : | 11,06 | 10,64 | 3,62 | copies |
| Master mix used : | Ruminant: qPCR 2x MasterMix Plus without UNG (Eurotentec); | | | |
| | Pig + Poultry: Agilent II QPCR with LowRox | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 4 | 28 | Present | Absent | Absent | |
| 3 | 79 | Present | Absent | Absent | |
| 5 | 535 | Present | Absent | Present | |
| 2 | 724 | Absent | Absent | Absent | |
| 1 | 739 | Absent | Present | Absent | |


## Laboratory identification code : **9**

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 36,64 | 37,16 | 37,04 | cycles |
| Copy number at the cut-off : | 10,32 | 3,76 | 9,46 | copies |
| Master mix used : | Brilliant II QPCR Low ROX Master Mix, cat. N. 600806 | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 1 | 325 | Absent | Present | Absent | the confirmatory method for the detection of specific animal species DNA was used (according to Köppel et al. (2009). EFRT, 230(1), 125-133.) - PRESENT chicken and turkey DNA |
| 4 | 442 | Present | Absent | Absent | the confirmatory method for the detection of specific animal species DNA was used (according to Köppel et al. (2009). EFRT, 230(1), 125-133.) - PRESENT bovine DNA |
| 2 | 472 | Absent | Absent | Absent | the confirmatory method for the detection of specific animal species DNA was used (according to Köppel et al. (2009). EFRT, 230(1), 125-133.) - ABSENT all of examined animal species DNA |
| 5 | 607 | Present | Absent | Present | the confirmatory method for the detection of specific animal species DNA was used (according to Köppel et al. (2009). EFRT, 230(1), 125-133.) - PRESENT bovine and porcine DNA |
| 3 | 835 | Present | Absent | Absent | the confirmatory method for the detection of specific animal species DNA was used (according to Köppel et al. (2009). EFRT, 230(1), 125-133.) - PRESENT ovine and goat DNA |


## Laboratory identification code : **10**

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 35,43 | 43,20 | 40,48 | cycles |
| Copy number at the cut-off : | 10,16 | 9,05 | 3,52 | copies |
| Master mix used : | Eurogenetec RT-QP2X-03 | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 4 | 46 | Present | Absent | Absent | |
| 5 | 139 | Present | Absent | Present | |
| 3 | 241 | Present | Absent | Absent | PCR inhibition |
| 1 | 703 | Absent | Present | Absent | |
| 2 | 922 | Absent | Absent | Absent | |

## Laboratory identification code : 11

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 36,47 | 37,32 | 39,12 | cycles |
| Copy number at the cut-off : | 11,46 | 10,75 | 4,00 | copies |
| Master mix used : | Eurogentec qPCR MasterMix RT-QP2X03-wou | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 2 | 256 | Absent | Absent | Absent | |
| 4 | 334 | Present | Absent | Absent | |
| 5 | 553 | Present | Absent | Present | |
| 3 | 763 | Present | Absent | Absent | |
| 1 | 829 | Absent | Present | Absent | |

## Laboratory identification code : 12

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 37,51 | 35,97 | 39,19 | cycles |
| Copy number at the cut-off : | 9,19 | 9,02 | 3,07 | copies |
| Master mix used : | Eurogentec RT-QP2X-03WOU+ | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 1 | 343 | Absent | Present | Absent | |
| 3 | 385 | Present | Absent | Absent | |
| 5 | 643 | Present | Absent | Present | |
| 4 | 658 | Present | Absent | Absent | |
| 2 | 958 | Absent | Absent | Absent | |

## Laboratory identification code : 13

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 34,61 | 38,40 | 38,93 | cycles |
| Copy number at the cut-off : | 9,18 | 9,25 | 3,06 | copies |
| Master mix used : | Eurogentec without UNG-EUK218 | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 4 | 388 | Present | Absent | Absent | |
| 5 | 409 | Present | Absent | Present | |
| 2 | 688 | Absent | Absent | Absent | |
| 3 | 745 | Present | Absent | Absent | |
| 1 | 919 | Absent | Present | Absent | |

## Laboratory identification code : 14

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 34,24 | 36,59 | 37,87 | cycles |
| Copy number at the cut-off : | 11,08 | 11,18 | 3,76 | copies |
| Master mix used : | Maxima Probe qPCR (x2) no BSA, no ROX | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 5 | 67 | Present | Absent | Absent | |
| 2 | 76 | Absent | Absent | Present | |
| 1 | 289 | Absent | Present | Absent | |
| 4 | 640 | Present | Absent | Absent | |
| 3 | 817 | Present | Absent | Absent | |

## Laboratory identification code : 15

|                                   | Ruminant | Poultry | Pig   |        |
|-----------------------------------|----------|---------|-------|--------|
| Cut-off at 15 (*5 for pig*) copies : | 34,66    | 37,31   | 39,32 | cycles |
| Copy number at the cut-off :      | 10,55    | 9,26    | 3,23  | copies |
| Master mix used : Universal Mastermix, „BIOSENSE ", Italy | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|-------------|-----------|--------------|-------------|---------|---------|
| 3 | 349 | Present | Absent  | Absent  | |
| 1 | 397 | Absent  | Present | Absent  | |
| 2 | 454 | Absent  | Absent  | Absent  | |
| 4 | 550 | Present | Absent  | Absent  | |
| 5 | 589 | Present | Absent  | Present | |

## Laboratory identification code : 16

|                                   | Ruminant | Poultry | Pig   |        |
|-----------------------------------|----------|---------|-------|--------|
| Cut-off at 15 (*5 for pig*) copies : | 32,23    | 35,67   | 36,96 | cycles |
| Copy number at the cut-off :      | 11,77    | 11,12   | 3,90  | copies |
| Master mix used : Eurogentec qPCR MasterMix without UNG | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|-------------|-----------|--------------|-------------|---------|---------|
| 3 | 7   | Present | Absent  | Absent  | |
| 2 | 328 | Absent  | Absent  | Absent  | |
| 1 | 379 | Absent  | Present | Absent  | |
| 5 | 499 | Present | Absent  | Present | |
| 4 | 856 | Present | Absent  | Absent  | |

## Laboratory identification code : 17

|                                   | Ruminant | Poultry | Pig   |        |
|-----------------------------------|----------|---------|-------|--------|
| Cut-off at 15 (*5 for pig*) copies : | 31,84    | 36,47   | 35,02 | cycles |
| Copy number at the cut-off :      | 13,97    | 13,74   | 4,71  | copies |
| Master mix used : qPCR MasterMix Eurogentec RT-QP2X-03 | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|-------------|-----------|--------------|-------------|---------|---------|
| 2 | 310 | Absent  | Absent  | Absent  | |
| 4 | 514 | Present | Absent  | Absent  | |
| 5 | 625 | Present | Absent  | Present | |
| 1 | 865 | Absent  | Present | Absent  | |
| 3 | 979 | Present | Absent  | Absent  | |

## Laboratory identification code : 18

|                                   | Ruminant | Poultry | Pig   |        |
|-----------------------------------|----------|---------|-------|--------|
| Cut-off at 15 (*5 for pig*) copies : | 35,75    | 37,65   | 40,04 | cycles |
| Copy number at the cut-off :      | 12,15    | 11,48   | 3,87  | copies |
| Master mix used : Brilliant II QPCR Low ROX Master mix Lot 6803777 | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|-------------|-----------|--------------|-------------|---------|---------|
| 4 | 100 | Present | Absent  | Absent  | Poultry DNA – sample 100 – during the first isolation showed inconsistent results (one isolate positive, one isolate negative). A new run of the first isolation gave the same result. New-isolation – both negative for poultry DNA. |
| 5 | 517 | Present | Absent  | Present | |
| 1 | 613 | Absent  | Present | Absent  | |
| 3 | 727 | Present | Absent  | Absent  | |
| 2 | 814 | Absent  | Absent  | Absent  | |

| Laboratory identification code : | **19** |
|---|---|

|  | Ruminant | Poultry | Pig |  |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 10,10 | 9,22 | 3,70 | cycles |
| Copy number at the cut-off : | 37,07 | 38,77 | 35,78 | copies |
| Master mix used : DMMLD2D600,Universal Mastermix , Biosense |  |  |  |  |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 3 | **133** | Present | Absent | Absent | |
| 5 | **211** | Present | Absent | Present | |
| 1 | **415** | Absent | Present | Absent | |
| 4 | **892** | Present | Absent | Absent | |
| 2 | **940** | Absent | Absent | Absent | |

| Laboratory identification code : | **20** |
|---|---|

|  | Ruminant | Poultry | Pig |  |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 34,90 | 36,99 | 37,75 | cycles |
| Copy number at the cut-off : | 9,17 | 9,04 | 3,40 | copies |
| Master mix used : LaCAR Universal mastermix |  |  |  |  |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 3 | **61** | Absent | Absent | Absent | |
| 2 | **544** | Absent | Absent | Absent | |
| 5 | **715** | Present | Absent | Present | |
| 1 | **721** | Absent | Present | Absent | |
| 4 | **964** | Present | Absent | Absent | |

| Laboratory identification code : | **21** |
|---|---|

|  | Ruminant | Poultry | Pig |  |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 35,08 | 35,38 | 36,90 | cycles |
| Copy number at the cut-off : | 10,96 | 9,35 | 3,65 | copies |
| Master mix used : RealQ Amplicon |  |  |  |  |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 1 | **73** | Absent | Present | Absent | |
| 5 | **175** | Present | Absent | Present | Signal just on LOD, but two times reanalysis gave  2x positive, or 1 pos and 1 neg. |
| 3 | **475** | Present | Absent | Absent | |
| 2 | **580** | Absent | Absent | Absent | |
| 4 | **622** | Present | Absent | Absent | |

| Laboratory identification code : | **22** |
|---|---|

|  | Ruminant | Poultry | Pig |  |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 37,40 | 45,00 | 42,00 | cycles |
| Copy number at the cut-off : | 9,14 | 9,02 | 3,11 | copies |
| Master mix used : AGILENT - Ref : 600806 |  |  |  |  |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 2 | **40** | Absent | Absent | Absent | |
| 5 | **247** | Present | Absent | Present | |
| 3 | **421** | Present | Absent | Absent | |
| 1 | **469** | Absent | Present | Absent | |
| 4 | **694** | Present | Absent | Absent | |

| Laboratory identification code : | 23 |
|---|---|

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 36,73 | 37,81 | 37,23 | cycles |
| Copy number at the cut-off : | 9,47 | 11,60 | 3,56 | copies |
| Master mix used : Agilent Brilliant II QPCR MasterMix with Low ROX | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 1 | 91 | Absent | Present | Absent | |
| 4 | 352 | Present | Absent | Absent | |
| 2 | 562 | Absent | Absent | Absent | |
| 3 | 583 | Present | Absent | Absent | |
| 5 | 967 | Present | Absent | Present | |

| Laboratory identification code : | 24 |
|---|---|

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 35,76 | 36,31 | 36,00 | cycles |
| Copy number at the cut-off : | 11,40 | 9,39 | 3,58 | copies |
| Master mix used : Eurogentec | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 2 | 274 | Absent | Absent | Absent | Was no signal at all (no ct value) for poultry and Porcine DNA |
| 3 | 313 | Present | Absent | Absent | Was no signal at all (no ct value) for poultry and Porcine DNA |
| 5 | 445 | Present | Absent | Present | Was no signal at all (no ct value) for poultry DNA |
| 4 | 496 | Present | Absent | Absent | Was no signal at all (no ct value) for poultry and Porcine DNA |
| 1 | 649 | Absent | Present | Absent | Was no signal at all (no ct value) for porcine DNA |

| Laboratory identification code : | 26 |
|---|---|

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 33,83 | 38,73 | 38,95 | cycles |
| Copy number at the cut-off : | 10,52 | 10,47 | 4,77 | copies |
| Master mix used : Brillant II qPCR Low Rox Master Mix | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 5 | 301 | Present | Absent | Present | |
| 3 | 457 | Present | Absent | Absent | |
| 1 | 577 | Absent | Present | Absent | |
| 4 | 676 | Present | Absent | Absent | |
| 2 | 706 | Absent | Absent | Absent | |

| | | Ruminant | Poultry | Pig | |
|---|---|---|---|---|---|
| Laboratory identification code : | **27** | | | | |

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 33,66 | | | cycles |
| Copy number at the cut-off : | 11,26 | | | copies |
| Master mix used : Eurogentec qPCR MasterMix Plus w/o UNG | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 3 | **25** | Present | Absent | Absent | 'The Poultry and Pig results were obtained using an In-House extraction and PCR. |
| 2 | **166** | Absent | Absent | Absent | 'The Poultry and Pig results were obtained using an In-House extraction and PCR. |
| 4 | **748** | Present | Absent | Absent | 'The Poultry and Pig results were obtained using an In-House extraction and PCR. |
| 1 | **775** | Absent | Present | Absent | 'The Poultry and Pig results were obtained using an In-House extraction and PCR. |
| 5 | **985** | Present | Absent | Absent | 'The Poultry and Pig results were obtained using an In-House extraction and PCR. |

| | | |
|---|---|---|
| Laboratory identification code : | **28** | |

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 37,29 | 37,27 | 39,65 | cycles |
| Copy number at the cut-off : | 11,04 | 10,94 | 3,86 | copies |
| Master mix used : "Brilliant II QPCR Low ROX Master Mix" reference number 600806 (Agilent, Santa Clara, CA, USA) | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 3 | **97** | Present | Absent | Present | |
| 1 | **127** | Absent | Present | Absent | |
| 4 | **586** | Present | Absent | Absent | |
| 5 | **751** | Present | Absent | Present | |
| 2 | **850** | Absent | Absent | Absent | |

| | | |
|---|---|---|
| Laboratory identification code : | **29** | |

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | 36,30 | 37,57 | 38,86 | cycles |
| Copy number at the cut-off : | 10,76 | 9,62 | 3,83 | copies |
| Master mix used : Brilliant II QPCR Low ROX Master Mix Agilent 600806 | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 5 | **49** | Present | Absent | Present | |
| 4 | **172** | Present | Absent | Absent | |
| 3 | **205** | Present | Absent | Absent | |
| 1 | **433** | Absent | Present | Absent | |
| 2 | **436** | Absent | Absent | Absent | |

| | Ruminant | Poultry | Pig | |
|---|---|---|---|---|
| Cut-off at 15 (*5 for pig*) copies : | | | | cycles |
| Copy number at the cut-off : | | | | copies |
| Master mix used : Taqman Fast Advance. Applied biosystems. Catálogo: 4444557. PCR Master Mix Promega. M7505. | | | | |

| Sample type | Sample N° | Ruminant DNA | Poultry DNA | Pig DNA | Comment |
|---|---|---|---|---|---|
| 2 | 148 | Absent | Absent | Absent | qPCR (Ruminant DNA 100 ng per reaction).  PCR-RFLP (poultry and pig) |
| 4 | 208 | Present | Absent | Absent | qPCR (Ruminant DNA 100 ng per reaction).  Ct cut off= 47.1. Ct detected= 44.29. PCR-RFLP (poultry and pig) |
| 1 | 595 | Absent | Present | Absent | qPCR (Ruminant DNA 100 ng per reaction).  PCR-RFLP (poultry and pig). Detected: Chicken |
| 5 | 679 | Present | Absent | Absent | qPCR (Ruminant DNA 100 ng per reaction).  Ct cut off= 47.1. Ct detected= 44.24.  PCR-RFLP (poultry and pig) |
| 3 | 691 | Present | Absent | Absent | qPCR (Ruminant DNA 100 ng per reaction).  Ct cut off= 47.1. Ct detected= 46.99. PCR-RFLP (poultry and pig) |