Combined microscopy-PCR
EURL-AP Proficiency Test 2023

Final version

Authors:

P. Veys, A. Marien & O. Fumière

March 2024

European Union Reference Laboratory for Animal Proteins in feedingstuffs
Walloon Agricultural Research Centre
Department Knowledge and Valorisation of Agricultural Products, Henseval Building, Chaussée de Namur 24,
B-5030 GEMBLOUX (BELGIUM)
Phone: +32 (0) 81 87 52 28 - Fax : +32 (0) 81 87 40 19
secretary@eurl.craw.eu – website: https://www.eurl.craw.eu

ISBN 978-2-87286-133-0
Legal Deposit D/2024/1463/1

Editor :

Centre wallon de Recherches agronomiques
Service Communication
Rue de Liroux, 9
5030 Gembloux (Belgique)

Table of content

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 participating laboratories was 32 (26 NRLs and 6 labs outside the NRL network). The study was based on a set of six 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 detection of fish material by light microscopy was improved as specificity problems identified during previous proficiency tests seem to be reduced with only three positive deviations accounting for 2 % of the results for this parameter. The detection of terrestrial vertebrates even at very low levels of adulteration is perfectly mastered by the network. For the very first time, the detection of terrestrial invertebrates was assessed as the other routine parameters. The sensitivity of the participants is perfect but the specificity has to be improved mainly by a correct application of the protocol and the conditions of implementation of the double PE/TCE sedimentation according to existing SOPs.

Concerning the PCR results, the participants were assessed on their proficiency to perform the three PCR tests for the detection of ruminant, pig and poultry DNA. Ninety-two % of the NRLs (24 out of 26) performed excellently and reported no false result. Two NRLs (8 %) were considered as underperforming: two positive deviations were recorded for the detection of ruminant DNA in one case whereas the second underperforming participant returned 2 negative deviations. The global performance of the network remains quite good with three PCR methods.

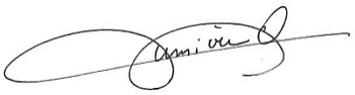
Keywords :

Processed animal proteins – Light microscopy – PCR - Proficiency test – Qualitative analysis

This report identified by an ISBN has been prepared from a draft version sent for revision and comments to the participants on the 27th February 2024. After reception and evaluation of the comments on the 12th March 2024, 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 (EURL) were created in order 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 organizing 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, the 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 (SOP) 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. A detailed list of the 32 participating labs is included in Annex 1.

Official announcement (Annex 2) of the study was made on the 8th September 2023 to all invited participants.

On the 13th October 2023, the sample sets were shipped to the participants (pick up of the samples by the transporter took however place on the 17th. Participants were informed on this shipment delay). On the 13th the Excel report forms containing the instructions (Annex 3) were also communicated to all participants - downloadable from the EURL-AP intranet for the NRLs or sent by email to the non-EU participants who have no access to this intranet.

The deadline for the delivery of the results was fixed in the announcement and in the instructions at the 17th November 2023.

Within the instructions, some general recommendations were delivered to the 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 3). Participants were asked to carefully read the instructions on how to fill in the result form and to testify they did it prior to encoding their results. No other support for communicating the results 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.

All results were delivered on time to the organiser. Thus, the study presents results from 32 participants. The 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. One test material was in duplicate. The composition of the sample set was established considering the following factors:

- Use of feed and feed materials intended to ruminant animals ;
- Absence of any ingredient from fish origin ;
- Use of pure ingredients from animal origin commercially available ;
- Use of a hairs from pet origin ;
- Use of insect material ;
- Use of adulterants from animal origins intended to deliver both positive presence for terrestrial vertebrates by light microscopy and positive ruminant and porcine signals by PCR.

Each participating lab received thus a sample set of six vials, each of about 40g, 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	Expected results * Microscopy (particles)			PCR (DNA)		
		Terrestrial vert.	Terrestrial invert.	Fish	Ruminant	Pig	Poultry
1	Bovine feed + 0.05 % bovine PAP	+	-	-	+	-	-
2	Ovine feed I + 1 % *T. molitor* PAP	-	+	-	-	n.a.*	-
3 + 4	Ovine feed II	-	n.a.*	-	-	-	-
5	Ovine feed II + 0.5 % pork blood meal + pet hairs	+	-	-	-	+	-
6	Ovine feed III + 0.01 % bovine PAP	+	-	-	+	-	-
Total positive results		**3**	**1**	**0**	**2**	**1**	**0**

(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 the samples (presence or absence of PAP) and the results obtained during the homogeneity study (see 3.5).

3.2.2. Materials used in the preparation of the samples

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

- The **bovine feed**, used to prepare sample 1 was a commercial complement feed for calves. It was composed of barley, corn, sunflower, soybean, corn germ extraction meal, wheat bran, wheat meal, partially hulled sunflower meal, beet pulp, calcium carbonate, molasses and sodium chloride. Its sediment content was of about 1.4 %. No animal remains could be detected by microscopy. The feed was slightly positive for ruminant DNA presence.
- The **ovine feed I** used in sample 2 was a complement feed for sheep and goats consisting of barley flakes, bran pellets, alfalfa granulates, maize flakes, linseed bran, barley grain and molasse. It also contained vitamins and mineral additives. Its sediment was of 1.3 %. The feed was slightly contaminated with weevil. No animal DNA was detected by PCR.
- The **ovine feed II**, used in duplicate in samples 3 and 4 and for preparing sample 5, was a complement feed for sheep, goats and deer. It was composed of barley flakes, malt rootlets, corn gluten feed, corn flakes, wheat bran, flax press, alfalfa and peas, beet pulp and molasses, calcium carbonate and dicalcium phosphate. Its sediment was of 1.5 %. No DNA from animal origin was detected.
- The last feed, **ovine feed III**, used for preparing sample 6, was a complement feed for sheep and lambs of which the composition was alfalfa, wheat semolina, barley, maize, wheat and corn gluten feed, palm kernel press cake, sunflower seed, linseed cake, hulled soybean, molasse and mineral complement. Its sediment was of 1.4 %. It was slightly positive for ruminant DNA.

Adulterant material used:

- A **ruminant PAP** was used for preparing samples 1 and 6. Its sediment content reached 60 %. Only ruminant DNA was detected by PCR.
- A **porcine blood meal** was used for preparing sample 5. It was free from sediment. PCR analyses revealed only the presence of porcine DNA.
- An **insect meal** was used for preparing sample 2. It was produced from the industry and consisted only of *Tenebrio molitor*, the mealworm. The insects were ground at 4 mm to produce a meal. No traces of terrestrial animal DNA could be detected by PCR.

- **Dog hairs** were collected to prepare sample 5. The hairs were powdered by using liquid nitrogen and a mortar.

3.2.3. Description of the mixing procedures

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

As a rule of best practice, all feed matrices were ground and conditioned separately. Only after the whole conditioning of the vials, adulterations were realised by direct spiking into the vials.

The ovine feed I (sample 2) was ground at 2 mm and conditioned 6 months before initiating the present study.

The ovine feed II (samples 3 and 4) was ground at 4 mm and conditioned in order to avoid contamination.

The ovine feed III (sample 6) was ground at 4 mm and conditioned.

The last conditioning concerned the bovine feed (sample 1).

Adulterations then followed.

The ovine feed II + 0.5 % pork blood meal + 0.1 % pet hairs (sample 4) was prepared from the 4 mm ground and conditioned matrix.

The ovine feed I (sample 2) vials were adulterated by spiking with the insect meal.

Then only adulteration with the bovine PAP was processed. First the bovine feed matrix (sample 1) and finally the ovine feed III matrix (sample 6)

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 in order 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-of 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, 5 and 6 had to be declared as positive.
- Samples 2, 3 and 4 had to be declared negative

Concerning the presence of terrestrial invertebrates:

- Sample 2 had to be declared as positive.
  Samples 1, 5 and 6 had to be declared negative

- Samples 3 and 4 were not submitted to proficiency assessment for this parameter. In the homogeneity study section of the report the explanation for this decision is detailed.

Concerning the presence of fish, all samples 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 > 0.85 without ND for terrestrial vertebrates and terrestrial invertebrates.
- **Underperforming** level of global performance: consolidated AC > 0.85 with one ND for terrestrial vertebrates or a consolidated AC ≤ 0.85.

3.4.2. PCR

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

- Samples 1 and 6 were considered to be declared positive for the presence of ruminant DNA.
- Samples 5 was considered to be positive for the presence of pig DNA.
- All samples were considered to be negative for the presence of poultry DNA.
- Samples 3 and 4 had to be declared negative.

The detection of pig 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 ≥ 0.94 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 ≥ 0.94 with 1 false result (ND or PD) for the detection of ruminant DNA or global AC < 0.94 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		Light microscopy			PCR			
		Nr of replicates	Terrestrial vert.	Terrestrial invert.	Fish	Nr of replicates	Ruminant	Porcine	Poultry
1	Bovine feed + 0.05 % bovine PAP	10	+	-	-	10	+	-	-
2	Ovine feed I + 1 % *T. molitor* PAP	10	-	+	-	10	-*	-	-
3 + 4	Ovine feed II	20	-	-*	-	20	-	-	-
5	Ovine feed II + 0.5 % pork blood meal + pet hairs	10	+	-	-	10	-	+	-
6	Ovine feed III + 0.01 % bovine 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]. For PCR analysis, a DNA extraction was performed on 2 test portions of 100 mg of sample material for each of the 10 replicates.

Through the light microscopic observations performed during the homogeneity study, no single fish particle could be identified through the whole sample set. It demonstrates a total absence of fish material.

Sample 1 (Bovine feed + 0.05 % bovine PAP) was systematically positive for the presence terrestrial vertebrates with the observations of bone fragments and few muscles. No traces of terrestrial invertebrates were detected although the replicates were not submitted to PE/TCE sedimentation. PCR analyses detected systematically the presence ruminant DNA whereas pig and poultry DNA were absent.

Sample 2 (Ovine feed I + 1 % *T. molitor* PAP) was free from any vertebrate particles. After PE/TCE sedimentation, each replicate showed an abundant presence of terrestrial invertebrates' fragments. No DNA from ruminant, pig and poultry was detected by PCR analyses on the items used for the homogeneity study. Nevertheless, the presence of ruminant DNA was sporadically detected during the preliminary preparation steps. For that reason, this parameter was kept out of the evaluation.

Samples 3 and 4 (Ovine feed II) were free from any vertebrates' presence. After the PE/TCE sedimentation, the majority of the replicates were negative for terrestrial invertebrates' fragments, except for 5 out of 20 replicates showing insect fragments at levels < LOD in their final flotates, representing a total of 7 particles over 80 slides. The criteria for the homogeneity study for this parameter was thus not satisfying and this parameter was excluded from proficiency assessment. No DNA from ruminant, pig and poultry was detected by PCR analyses.

Table 3: Homogeneity study – Details insect fragments on samples 3 and 4

Number of slides	Number of terrestrial invertebrates' fragments	Total
flotate (TCE) : 40	0	0
final flotate (PE/TCE) : 80	2 + 2 + 1 + 1 + 1	7

Sample 5 (Ovine feed II + 0.5 % pork blood meal + pet hairs) was systematically positive for the presence terrestrial vertebrates' fragments identified as blood and hairs. No traces of terrestrial invertebrates could be detected although, with respect to the conditions of the SOP on the combination of methods, the replicates were not submitted to PE/TCE sedimentation. Porcine DNA was systematically detected by PCR analyses. Neither ruminant nor poultry DNA was present.

Sample 6 (Ovine feed III + 0.01 % bovine PAP) was systematically positive for the finding of terrestrial vertebrates' remains. Due to the low adulteration level, slides prepared showed no more than 5 bones per slides and muscles were very scare. PCR analyses detected systematically the presence ruminant DNA whereas porcine and poultry DNA were absent.

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. There is no reasonable element which would indicate that present samples should be unstable.

4. Results

Gross results for microscopy and PCR from all participants are to be found in Annexes 4 and 5 respectively.

4.1. Microscopy results

4.1.1. Qualitative analyses from the NRLs

4.1.1.1. Results and performance of the network

Table 4 summarizes the results reported by the 26 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 5 % of the total responses.

Table 4: Global results expressed as accuracy (AC) – light microscopy

Sample	Material	n	AC		
			Terrestrial vert.	Terrestrial invert.	Fish
1	Bovine feed + 0.05 % bovine PAP	26	1.000	0.960 (1)	0.923 (2)
2	Ovine feed I + 1 % *T. molitor* PAP	26	0.962 (1)	1.000	1.000
3 + 4	Ovine feed II	52	0.981 (1)	n.a.	0.981 (1)
5	Ovine feed II + 0.5 % pork blood meal + pet hairs	26	1.000	0.720 (7)	1.000
6	Ovine feed III + 0.01 % bovine PAP	26	1.000	0.920 (2)	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, n.a. = not submitted to proficiency assessment).

Since the absence of any fish material within the current study, as it was in the past study in 2022 [6] only false positive results could be considered for calculating specificity scores. Only three specificity issues were noted within the collected results of the NRL network. This represents only 2 % of error for this parameter.

Regarding the detection of terrestrial vertebrates' constituents, the sensitivity was perfect without any false negative results and the specificity did not revealed any major issue since only two false positive cases were reported (one for sample 2 and one for sample 3). This accounts for only 1 % of error for this parameter.

The detection of terrestrial invertebrates on a routine way within the scope of an EURL-AP proficiency assessment is a first. Results demonstrated a perfect sensitivity score for insect particles. Problems of specificity were noted, ranked by number of false positive results, for sample 1, 6 and 5. They accounted for the major source of error in the present study or 10% for this parameter.

Further details on the errors noted in this study are in described in next point. Possible keys to the problems are presented in the discussion section, especially regarding sample 5 which deserves clarification.

4.1.1.2. Detailed review of results per sample

Sample 1: Bovine feed + 0.05 % bovine PAP

All results were correct for the terrestrial vertebrate parameter.

PD for terrestrial invertebrates' particles:

- Lab 11 reported chitin.

Lab 23 didn't answer to this parameter although instructions were specifying that leaving blanks was not allowed.

PD for fish particles:

- Lab 8 reported bones from fish origin.
- Lab 20 classified the sample as positive for fish without any description of the observed particles.

Sample 2: Ovine feed I + 1 % *T. molitor* PAP

PD for terrestrial vertebrates' particles:

- Lab 25 reported the presence of bones.

All results were correct for the terrestrial invertebrates and fish parameters

Samples 3 and 4: Ovine feed II

PD for terrestrial vertebrates' particles:

- Lab 11 reported the presence of plasma in one of the two replicates (sample 3).

PD for fish particles:

- Labs 22 reported erroneously the presence of fish scales.

This material showed during the homogeneity study, for 5 out of 20 replicates, a few insect particles after double PE/TCE sedimentations, each time at levels < LOD as reported on table 3. Although out of performance evaluation, the following results for terrestrial invertebrates were obtained by the NRL network, all after having performed a double PE/TCE sedimentation:

Table 5: Share of different results for samples 3 and 4

Negative 2x	Negative and < LOD	Negative and positive	Positive 2x
10 labs	3 labs	7 labs	6 labs
Descriptions of particles*			
	setae, insect	anal spine, leg, insect	tracheal system setae (4x) head, legs, mandibula, mouth parts (2x) denticle-like structures, insect appendages (2x)

*** out of poorly describing wordings such as chitin, cuticle fragments or cuticular structures.**

Sample 5: Ovine feed II + 0.5 % pork blood meal + pet hairs

All results were correct for the terrestrial vertebrate parameter. Blood and hair were detected allowing the sample to be declared positive for terrestrial vertebrates and thus terminating the analyses in accordance with the SOP on the combination of methods, thus not allowing a double PE/TCE sedimentation to be carried out.

PD[†] for terrestrial invertebrates' particles:

- Labs 2, 3, 6, 9, 11, 15 and 22 declared the presence of insects after having performed unauthorised PE/TCE sedimentation.

Lab 14 observed insect cuticles at a level < LOD.

Lab 23 didn't answer to this parameter although instructions were specifying that leaving blanks was not allowed.

[†] In the specific case of sample 5, the term PD is only used in relation with the expected NA. The finding of terrestrial invertebrates' fragments is a possible bias following an unrequired PE/TCE sedimentation as a preparation step.

Sample 6: Ovine feed III + 0.01 % bovine PAP

Again, results were correct for the terrestrial vertebrate presence and thus terminating the analyses in accordance with the SOP on the combination of methods, thus not allowing a double PE/TCE sedimentation to be carried out.

PD for terrestrial invertebrates' particles:

- Labs 4 and 11 declared the presence of cuticles after having performed unauthorised PE/TCE sedimentation.

Lab 17 reported insect particles at a level < LOD.

Lab 23 didn't answer to this parameter although instructions were specifying that leaving blanks was not allowed.

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 6, 7 and 8. A ranking of the labs was prepared based on the consolidated accuracy.

Tables 6 (left) and 7 (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, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 26	1.000	1.000	1.000
11 and 25	0.833	1.000	0.667

Terrestrial inv.			
lab code	**AC**	**SE**	**SP**
1, 5, 7, 8, 10, 12, 13, 14, 16, 17, 18, 19, 20, 21, 24, 25 and 26	1.000	1.000	1.000
2, 3, 4, 6, 9, 15 and 22	0.750	1.000	0.667
11 and 23	0.250	1.000	0.000

Table 8: NRL proficiencies regarding the detection of fish material. Ranking follows AC values for primary key and SP for second key

Fish		
lab code	**AC**	**SP**
1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,	1.000	1.000

21, 23, 24, 25 and 26		
8, 20 and 22	0.833	0.833

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 9).

Table 9: 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**
1, 5, 7, 10, 12, 13, 14, 16, 17, 18, 19, 21, 24 and 26	1.000	1.000	1.000
2, 3, 4, 6, 8, 9, 15, 20 and 25	0.938	1.000	0.917
22	0.875	1.000	0.833
23	0.813	1.000	0.750
11	0.750	1.000	0.667

From the 26 NRLs, 14 performed excellently (54 %), 10 performed satisfyingly (38 %) and 2 were underperforming (8 %).

In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), the underperforming participants (labs 11 and 23) are asked to report on the origin of their errors as well as on the actions they will undertake in order to solve the problems.

4.1.2. Qualitative analyses and individual performances the non-EU participants

Individual performances from the 6 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 10, 11 and 12.

Tables 10 (left) and 11 (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**
30 and 32	1.000	1.000	1.000
27 and 29	0.833	0.667	1.000
28	0.667	0.667	0.667
34	0.667	0.333	1.000

Terrestrial inv.			
lab code	**AC**	**SE**	**SP**
30	1.000	1.000	1.000
34	0.750	1.000	0.667
27 and 29	0.750	0.000	1.000
32	0.250	1.000	0.000
28	0.250	0.000	0.333

Table 12: 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**	**SP**
28, 30 and 32	1.000	1.000
27 and 29	0.667	0.667
34	0.500	0.500

Sample 1: Bovine feed + 0.05 % bovine PAP

ND for terrestrial vertebrates' particles:

- Lab 34 failed at detecting terrestrial vertebrates

Lab 27 only detected bones < LOD.

PD for terrestrial invertebrates' particles:

Lab 32 didn't answer to this parameter although instructions were specifying that leaving blanks was not allowed.

PD for fish particles:

- Lab 34 reported bones from fish origin.

Sample 2: Ovine feed I + 1 % *T. molitor* PAP

ND for terrestrial invertebrates' particles:

- Labs 27, 28 and 29 failed at detecting insect fragments.

PD for fish particles:

- Lab 34 erroneously identified fishbones.

Samples 3 and 4: Ovine feed II

PD for terrestrial vertebrates' particles:

- Lab 28 reported the presence of bones in one of the two replicates (sample 3).while for sample 4 this presence was < LOD.

PD for fish particles:

- Labs 27 and 29 reported erroneously the presence of fishbones and scales in one of the two replicates.

For the detection of terrestrial invertebrates, this sample was out of performance evaluation. The results delivered by non-EU participants were consisting of 4 positive declarations (but 2 out of them with a detailed description of bones, which is obviously an error), 7 negative declarations and 1 < LOD. Different sedimentation methods were reported: double PE/TCE sedimentation (6), single TCE sedimentation (4) and chloroform (2).

Sample 5: Ovine feed II + 0.5 % pork blood meal + pet hairs

ND for terrestrial vertebrates' particles:

- Lab 28 failed at detecting the blood and hairs

PD for terrestrial invertebrates' particles:

- Lab 28 declared the sample as positive for this parameter while describing bones (this is indicating a possible erroneous result encoding).

Lab 32 did not deliver a result for this parameter.

PD for fish particles:

- Labs 27 and 29 erroneously described fish presence.

Sample 6: Ovine feed III + 0.01 % bovine PAP

ND for terrestrial vertebrates' particles:

- Lab 34 failed at detecting terrestrial vertebrates

Lab 29 reported bones at a level < LOD.

PD for terrestrial invertebrates' particles:

- Lab 28 declare the sample as positive but detailing bones.
- Lab 34 declared the presence of cuticles.

Lab 32 didn't answer to this parameter.

PD for fish particles:

- Lab 34 reported erroneously the presence of fishbones.

A general ranking as for the NRL network was established (Table 13).

Table 13: General non-EU lab proficiency. Ranking follows AC values as primary key and SE as second key. Lines in black refer to excellent results, lines in blue refer to satisfying results and lines in red refer to underperforming results

Consolidated			
lab code	AC	SE	SP
30	1.000	1.000	1.000
32	0.813	1.000	0.750
27 and 29	0.750	0.500	0.833
28	0.688	0.500	0.750
34	0.625	0.500	0.667

One participant performed excellently and five 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 seem to stick generally to the SOPs. Nevertheless, very few labs do not use one of the EURL-AP recommended mastermixes : Maxima Probe qPCR Master Mixes - Thermo Scientific (Lab 03), TaqMan™ Universal PCR Master Mix - Applied Biosystems™ (Labs 14 and 23), SensiFAST™ Probe No-ROX Kit-bioline meridian bioscience® (Lab 22). Lab 23 uses the TaqMan™ Universal PCR Master Mix for the detection of ruminant DNA whereas it uses the Brilliant II QPCR Low ROX Master Mix from Agilent for the detection of pig and poultry DNA.

4.2.1.2. Overview of results and global performance of the network

Table 14 (next page) summarizes the results provided by 26 NRLs for the six samples submitted to qualitative PCR analysis.

Table 14: Global results expressed as accuracy (AC) – PCR

Sample	Material	n	AC		
			Ruminant	Pig	Poultry
1	Bovine feed + 0.05 % bovine PAP	26	0.962 (1)	1.000	1.000
2	Ovine feed I + 1 % *T. molitor* PAP	26	n.a.	1.000	1.000
3 + 4	Ovine feed II	52	0.981 (1)	1.000	1.000
5	Ovine feed II + 0.5 % pork blood meal + pet hairs	26	0.962 (1)	1.000	1.000
6	Ovine feed III + 0.01 % bovine PAP	26	0.962 (1)	1.000	1.000

Accuracy means sensitivity in case of ND and specificity in case of PD.
The absence of a PCR result is considered as a deviation (ND or 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 (0.9 % of the 442 results) were recorded. With the porcine and the poultry targets, the results are perfect. The rate of false results obtained with the ruminant assay is 3.1 % which remains acceptable.

Sample 1 : Bovine feed + 0.05 % bovine PAP

The PCR results expected were the presence of ruminant DNA only. One negative deviation for the detection of ruminant DNA was recorded (Lab 20). All the results for the detection of porcine and poultry DNA were correct.

Sample 2 : Ovine feed I + 1 % T. molitor PAP

The PCR results expected were the absence of ruminant, pig and poultry DNA. No deviation recorded with the porcine and poultry assays. The results for the detection of ruminant DNA were kept out of the assessment but it must be noticed that all the EU participants reported the sample negative for this parameter.

Samples 3 and 4 : Ovine feed II

The PCR results expected were the absence of ruminant, pig and poultry DNA. One positive deviation for the detection of ruminant DNA was recorded (Lab 08). All the results for the detection of porcine and poultry DNA were correct.

Sample 5 : Ovine feed II + 0.5 % pork blood meal + pet hairs

The PCR results expected were the presence of pig DNA and the absence of ruminant and poultry DNA. One positive deviation for the detection of ruminant DNA was recorded (Lab 08). All the results for the detection of porcine and poultry DNA were correct.

Sample 6 : Ovine feed III + 0.01 % bovine PAP

The PCR results expected were the presence of ruminant DNA only. One negative deviation for the detection of ruminant DNA was recorded (Lab 20). All the results for the detection of porcine and poultry DNA were correct.

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 15 (next page) that summarizes the results obtained by the participants.

Table 15: 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, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23, 24, 25 and 26	1.000	1.000	1.000
8	0.882	1.000	0.857
20	0.882	0.333	1.000

Excellent performances were recorded for 24 labs out of 26 NRLs (77 % of the NRLs) having no false result.

Two labs (Labs 08 and 20) are underperforming. Two positive deviations were recorded by Lab 08 for the detection of ruminant DNA whereas the second underperforming participant (Lab 20) returned 2 negative deviations for the same test.

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.

All the participants reached the minimum criterion of 9.00 copies for the ruminant cut-off. The range of copies at the cut-off goes from 9.00 copies to 12.81 copies. The cut-off in cycles are comprised between 30.62 cycles and 37.84 cycles. The percentage of the labs with a cut-off corresponding to a number of copies > 10 for this proficiency test was 80.7 %.

For the pig cut-off too, all the participants reached the minimum criterion set at 3.00 copies. The range of copies at the cut-off goes from 3.05 copies to 4.58 copies. The cut-off in cycles are comprised between 34.18 cycles and 41.37 cycles. The percentage of the labs with a cut-off corresponding to a number of copies > 3.50 for this proficiency test was 53.8 %.

Considering the poultry cut-off, one NRL did not reach the minimum criterion of 9.00 copies. Nevertheless, it did not impact their results as no deviation was recorded. The range of copies at the cut-off goes from 7.92 copies to 11.96 copies. The cut-off in cycles are comprised between 35.83 cycles and 43.20 cycles.

It must be noticed that some participants used the same cut-offs as for the PT 2022.

4.2.2. Qualitative analyses from the non-EU participants

4.2.2.1. Individual performances

Individual performances were assessed for four 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 16.

Table 16 : 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 and 30	1.000	1.000	1.000
32	0.941	0.667	1.000
27	0.824	0.333	0.929

Labs 29 and 30 obtained excellent results (no deviation).

For Lab 32, one negative deviation for the detection of pig DNA was recorded with sample 5 (ovine feed containing 0.5 % of pork blood meal and pet hairs).

Lab 27 reported two negative deviations for the detection of ruminant DNA with samples 1 and 6. It reported also a positive deviation for pig DNA in sample 3.

4.2.2.2. Assessment of the cut-off values

Lab 27 does not use EURL-AP PCR methods and no cut-off value was indicated by the participant.

Labs 29 and 30 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.

Concerning Lab 32, the criterion is reached for the ruminant test but data were missing for the pig and the poultry tests.

5. Discussion and conclusions

The present study was organised with focus on several factors which were mentioned in the description of the samples.

First of all, this year again, fish material was excluded from the study. The purpose was to control the baseline specificity problems depicted from last year study [6]. Results obtained demonstrated an improvement of the NRL network since only three errors, accounting for only 2 %, for this parameter were reported. This represent a major progress compared to the past 2022 study [6].

Secondly, the detection of terrestrial vertebrate particles, either in presence of classical PAP containing bones and muscles, or in presence of less characterised materials such as blood meal and hairs, occurred faultless within the NRL network. Low adulteration levels, at 0.05 % and 0.01 % w/w, did not influence the results which were perfect in terms of sensitivity. The unusual presence of pet hairs, mimicking an environmental contamination at a level of 0.1 % w/w, did neither cause any specific problem: twenty-five NRLs mentioned in the details of their observations the finding of hairs or hair fragments. At large, specificity issues for terrestrial vertebrates' particles were also very limited.

Thirdly the main innovation is that for the very first time since the organisation of EURL-AP proficiency tests, terrestrial invertebrates' presence or absence was assessed on a routine way. No specific instructions were given to the participants in this regard. The SOP on the combination of methods had simply to be followed based on the labels on the sample vials - all referring to ruminant feeds. The participants were thus questioned to choose or not the double PE/TCE sedimentation based on the findings from the mandatory initial single TCE sedimentation.

About the detection capabilities, the sensitivity for terrestrial invertebrates' detection was perfect which was not the case for the specificity. If some minor false positive results were reported for the feeds adulterated with the bovine PAP, no clear explanation could be found. A situation which was entirely different regarding the errors observed for sample 5, based on the ovine feed matrix used also for samples 3 and 4 (duplicates). The homogeneity revealed that a few insect fragments, each time at a level below the limit of decision, could be detected in this feed after double PE/TCE sedimentations only, whereas in the case of single TCE sedimentations all of the sample replicates were negative. Out of the proficiency assessment, the results obtained by the participants for samples 3 and 4, submitted to double sedimentations, were correspondingly illustrating the inconsistency and the absence of repeatability and/or reproducibility for this parameter: 56 % of negative, 39% of positive findings and 5 % of < LOD findings. According to the SOP on the combination of methods, a double PE/TCE sedimentation is only authorized when from a first TCE sedimentation a feed or feed material intended for ruminant is negative for terrestrial vertebrates PAP or blood products. Now considering the results of sample 5, the ovine feed fortified with blood and hairs, all NRLs participants declared correctly the sample as positive for terrestrial vertebrates, thus ending the testing protocol in accordance with the SOP on the combination of methods. Nevertheless, this condition was only respected by 2 NRLs. The twenty-four other NRLs choose for an unauthorised additional PE/TCE sedimentation which eventually led them to reporting (7 positive declarations on a total of 15 negative ones and 1 < LOD) deviating from the one based on a single TCE sedimentation. This situation repeated on sample 1 and 6 for which all erroneous positive findings were biases due to an unnecessary PE/TCE sedimentation. One more time it is demonstrating that a strict respect of the protocol, including the SOP on the combination of methods, is important for harmonisation of the implementation of the methods.

The detailed description of the insect particles from samples 3 and 4 are also delivering interesting elements. The reporting of fragments identified by some NRLs as legs, insects (interpreted as entire insects), heads, mandibula, mouth parts and appendages can preferentially be associated with the presence of imagos rather than larvae from which PAPs are produced. Such information could enable to discriminate between terrestrial invertebrates' PAP adulteration and natural infestation where insects, at different developmental stages, are common as commented by the literature [7] and as added in last update of the SOP (V.5.1).

In 2023, a proficiency test organised by the IAG, was the first large scale proficiency assessment involving the new terrestrial invertebrates' parameter with an obtained sensitivity of 0.83 [8]. The later study concluded that this score for insect detection would be improved in the future. The present study showed a perfect score for the sensitivities, not only for insects, but also for other terrestrial vertebrates PAPs. It demonstrates the acquired expertise at EU level for this parameter.

To conclude on the microscopic results, even if the introduction of the new parameter terrestrial invertebrates was challenging, the overall scores are better than the ones from past two years [6, 9]. The number of excellent and satisfactory scores obtained within the network of NRLs reached respectively 54 % (39 % in 2022 and 34.5 % in 2021) and 38 % (42 % in 2022 and 31 % in 2021). The rate of underperforming NRL for the present study reached 8 % (19 % in 2022 and 34.5 % in 2021). Dedicated follow-up actions will be undertaken for each of them.

Concerning results from the six non-EU participants, encountered problems were different. Among them, the most frequent sources of error are specificity issues for fish and both specificity and sensitivity issues for terrestrial invertebrates. Such situation could effectively be anticipated as a majority of them are not bound to the same legal framework and are not trained in the detection of terrestrial invertebrates' PAPs.

Like in 2022, the performances of the NRL network were assessed with the three PCR tests (ruminant, pig and poultry) validated and implemented in the network. All the samples had to be analysed by PCR independently of the light microscopy results. Consequently, the PCR results reflect tangibly the real performances of the participants obtained with these methods.

The PCR skill of the NRLs network specifically with the ruminant PCR method continues to be confirmed. A higher number of deviations is observed (4 deviations - instead of 1 in 2022 - representing 3.1 % of the results) but these deviations are concentrated in only two labs. This means that 92 % of the NRLs perfectly managed the analyses of the set of samples.

The performances of the network are perfect with the pig and the poultry PCR methods. No deviation was recorded.

The individual performances of the participants are optimistic. Twenty-four NRLs out of 26 (92 %) returned results without any deviation. Two labs had two deviations: for one of them, there were 2 positive deviations with the ruminant test while for the other lab, 2 negative deviations were recorded.

One result was kept out of assessment by the organisers due to divergent results obtained during the preliminary steps of the preparation of the samples homogeneity study. Nevertheless, all the participants came to the same conclusion as the organisers: absence of ruminant DNA in the sample 2. Fundamentally, the removing of this parameter does not change the excellent performances of the network.

Acknowledgment

We are grateful to the EURL-AP technical staff for their preparation work and the efforts made to meet the ISO 17043 requirements: A. Cordonnier, L. Plasman, J. Maljean and C. Aerts. We also thank the participants for their fruitful collaboration.

References

[1] EU. 2017. Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation). Official Journal of the European Union L 95, 7/4/2017: 1-142.

[2] EU. 2011. Commission Regulation (EU) No 208/2011 of 2 March 2011 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council and Commission Regulations (EC) No 180/2008 and (EC) No 737/2008 as regards lists and names of EU reference laboratories. Official Journal of the European Union L 58, 3/3/2011: 29–35.

[3] EU. 2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Official Journal of the European Union L 54, 26/2/2009: 1-130.

[4] EU. 2013. Commission Regulation (EU) No 56/2013 of 16 January 2013 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. Official Journal of the European Union L 21, 24/1/2013: 3-16.

[5] ISO 13528:2015, Statistical methods for use in proficiency testing by interlaboratory comparison.

[6] Fumière O., Veys P. and Marien A. 2023. Combined microscopy-PCR EURL-AP Proficiency Test 2022: Final version. CRA-W, Gembloux, Belgium.

[7] Veys P. and Baeten V. 2018. Protocol for the isolation of processed animal proteins from insects in feed and their identification by microscopy. Food Control, 92, p 496-504.

[8] Veys P and Fumière O. 2023. 2023 IAG proficiency test on animal proteins in feed. CRA-W, Gembloux, Belgium.

[9] Veys P., Fumière O. and Marien A. 2022. Combined microscopy-PCR EURL-AP Proficiency Test 2021: Final version. CRA-W, Gembloux, Belgium.

Annex 1

List of participants (Laboratories that do not belong to the NRL network are *in italics*).

Country	Institute Name
Argentina	*SENASA*
Austria	Austrian Agency for Health and Food Safety
Belgium	Federal Agency for the Safety of the Food Chain
Bulgaria	National Diagnostic Research Veterinary Medical Institute
China	*China Agricultural University*
Croatia	Croatian Veterinary Institute
Cyprus	Cyprus Veterinary Services
Czech Republic	State Veterinary Institute Jihlava
Denmark	Ministry of Food, Agriculture and Fisheries Danish Veterinary and Food Admin
Estonia	National Centre for Laboratory Research and Risk Assessment (LABRIS)
Finland	Finnish Food Safety Authority
France	DG for Fair Trading, Consumer Affairs and Fraud Control-Laboratory Directorate Rennes
Germany	Federal Institute for Risk Assessment
Greece	Feedstuffs Control Laboratory of Thessaloniki
Hungary	National Food Chain Safety Office, Food and Feed Safety Directorate, Analytical National Reference Laboratory
Ireland	Department of Agriculture and Food Microscopy Laboratory - Seed Testing Station
Italy	National Reference Centre for the Surveillance and Monitoring of Animal Feed
Latvia	Institute of Food Safety, Animal Health and Environment (BIOR)
Lithuania	National Food and Veterinary Risk Assessment Institute
Luxemburg	Agroscope Liebefeld-Posieux Research Station (Switzerland)
Netherlands	Wageningen Food Safety Research
Norway	*Institute of Marine Research*
Poland	National Veterinary Research Institute
Portugal	Instituto Nacional de Investigacao Agraria e Veterinaria
Romania	Hygiene Institute of Veterinary Health
Serbia	*Institute of Veterinary Medicine of Serbia*
Slovakia	State Veterinary and Food Institute
Slovenia	Veterinary faculty - National Veterinary Institute - Institute of Food Safety, Feed and Environment - Department of Environment, Animal Nutrition, Welfare and Hygiene
Spain	Laboratorio Arbitral Agroalimentario
Sweden	National Veterinary Institute, Department of Animal Feed
Thailand	*Bureau of Quality Control of Livestock Products*
United Kingdom	*Animal and Plant Health Agency*

Annex 2

Announcement letter

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Knowledge & valorisation of Agricultural Products Department

Henseval building
Chaussée de Namur 24, B – 5030 GEMBLOUX

☎32 (0) 81 87 52 28 ✉32 (0) 81 87 40 19
e-mail: secretary@eurl.craw.eu Internet : https://www.eurl.craw.eu

Announcement of the EURL-AP proficiency test 2023/01 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 6 samples, each of about 40g. Each sample 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.
- The participation in this proficiency study is mandatory and free of charge for national reference laboratories within Member States of the European Union.

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Knowledge & valorisation of Agricultural Products Department

Henseval building
Chaussée de Namur 24, B – 5030 GEMBLOUX

☎32 (0) 81 87 52 28 🖷32 (0) 81 87 40 19
e-mail: secretary@eurl.craw.eu Internet : https://www.eurl.craw.eu

Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : 8 September 2023
- Sending of the sample boxes and communication of the instructions : 13 October 2023

> ***By default, samples will be sent to the NRL microscopy contact person 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 : 17 November 2023

Further information

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

or

- Dr Pascal VEYS
  EURL-AP NRL Network Manager
  ☎32 (0) 81 87 52 28
  🖷32 (0) 81 87 40 19
  E-mail: p.veys@cra.wallonie.be

or

- Dr Olivier FUMIERE
  Head of EURL-AP Molecular biology team
  ☎32 (0) 81 87 52 40
  🖷32 (0) 81 87 40 19
  E-mail: o.fumiere@cra.wallonie.be

Annex 3

Excel result report form

Proficiency Test Microscopy-PCR 2023/01

Laboratory identification

Laboratory code : 1

Responsibility agreement : No

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

Report

Lab code	1	1	1	1	1	1
Sample rank	1st	2nd	3th	4th	5th	6th
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.*						
Sedimentation(s)						
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						
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						
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						
Free comment *Example : PCR inhibition,…*						

Annex 4

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.	Fractions used	Number of determinations
6	47	Present	bones	Absent		Absent		PE/TCE	2
3	173	Absent		Absent		Absent		PE/TCE	2
1	193	Present	bones	Absent		Absent		PE/TCE	2
2	351	Absent		Present	cuticule	Absent		PE/TCE	2
5	465	Present	hair,blood	Absent		Absent		PE/TCE	2
4	559	Absent		Present	cuticule	Absent		PE/TCE	2

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.	Fractions used	Number of determinations
2	3	Absent		Present	Cuticule,'Tracheal system, setae	Absent		PE/TCE	1
1	49	Present	bones, muscle fibers	Absent		Absent		PE/TCE	1
6	59	Present	bones, muscle fibers	Absent		Absent		PE/TCE	1
3	77	Absent		Present	Setae, head, leg, mandibula	Absent		PE/TCE	1
4	475	Absent		Present	Tracheal system, setae, cuticule	Absent		PE/TCE	1
5	525	Present	blood products, hair, muscle fiber,	Present	'Tracheal system, setae, cuticule	Absent		PE/TCE	2

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.	Fractions used	Number of determinations
3	5	Absent		Present	Leg, cuticule	Absent		PE/TCE	2
6	83	Present	bones	Absent		Absent		PE/TCE	2
2	159	Absent		Present	cuticule	Absent		PE/TCE	2
1	229	Present	bones	Absent		Absent		PE/TCE	2
5	369	Present	blood, hairs	Present	cuticule	Absent		PE/TCE	2
4	451	Absent		Absent		Absent		PE/TCE	2

Tetramethylbenzidine - Hydrogen peroxide were used as a mounting medium for detecting blood in flotate.

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.	Fractions used	Number of determinations
2	87	Absent		Present	sensilla, cuticula	Absent		PE/TCE	2
6	167	Present	bone	Present	cuticula, muscle	Absent		PE/TCE	2
1	241	Present	bone	Absent		Absent		PE/TCE	2
4	427	Absent		Absent		Absent		PE/TCE	1
3	461	Absent		Absent		Absent		PE/TCE	1
5	513	Present	blood (haemoglobin) hair	Absent		Absent		PE/TCE	2

Laboratory identification code :**5**

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.	Fractions used	Number of determinations
6	95	Present	bones	Absent		Absent		PE/TCE	1
1	217	Present	bones, muscles	Absent		Absent		PE/TCE	1
2	243	Absent		Present	cuticule particles, muscles	Absent		PE/TCE	1
3	449	Absent		Absent		Absent		PE/TCE	1
4	523	Absent		Present	cuticule particles, anal spine	Absent		PE/TCE	1
5	609	Present	blood, hairs	Absent		Absent		PE/TCE	1

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.	Fractions used	Number of determinations
6	35	Present	bone	Absent		Absent		PE/TCE	1
1	97	Present	bone	Absent		Absent		PE/TCE	1
4	115	Absent		Present	setae, cuticular structures	Absent		PE/TCE	1
5	177	Present	hair	Present	setae, cuticular structures	Absent		PE/TCE	1
2	315	Absent		Present	setae, cuticular structures	Absent		PE/TCE	1
3	437	Absent		Present	setae, cuticular structures	Absent		PE/TCE	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.	Fractions used	Number of determinations
4	175	Absent		Absent		Absent		PE/TCE	1
1	205	Present	Bone fragments	Absent		Absent		PE/TCE	2
2	267	Absent		Present	Leg, cuticole fragments, trachea	Absent		PE/TCE	2
6	539	Present	Bone fragments	Absent		Absent		PE/TCE	2
5	561	Present	Hairs	Absent		Absent		PE/TCE	2
3	617	Absent		Absent		Absent		PE/TCE	1

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.	Fractions used	Number of determinations
2	147	Absent		Present	cuticule fragments, setae	Absent		PE/TCE	1
6	299	Present	bones	Absent		Absent		TCE	2
3	305	Absent		< LOD	cuticule fragment, setae	Absent		PE/TCE	2
1	445	Present	bones	Absent		Present	fishbones	TCE	2
5	453	Present	blood meal	Absent		Absent		TCE	2
4	607	Absent		Absent		Absent		PE/TCE	1

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.	Fractions used	Number of determinations
2	27	Absent		Present	cuticle fragments, muscles, tracheal structures, denticle-like structure	Absent		PE/TCE	1
3	89	Absent		Present	cuticle fragments, denticle-like structures, hair-like structures	Absent		PE/TCE	1
1	121	Present	bones, muscles	Absent		Absent		PE/TCE	1
6	335	Present	bones	Absent		Absent		PE/TCE	1
4	499	Absent		Present	cuticle fragments, mouthpart	Absent		PE/TCE	1
5	501	Present	hair, blood	Present	cuticle fragments, headpart, muscles, hair-like structures	Absent		PE/TCE	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.	Fractions used	Number of determinations
1	25	Present	bones	Absent		Absent		PE/TCE	2
6	131	Present	bones	Absent		Absent		PE/TCE	2
5	381	Present	hair, blood particles	Absent		Absent		PE/TCE	2
3	413	Absent		Absent		Absent		PE/TCE	1
4	415	Absent		Absent		Absent		PE/TCE	1
2	507	Absent		Present	cuticula, muscles	Absent		PE/TCE	2

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.	Fractions used	Number of determinations
1	1	Present	Bone, muscle	Present	Chitin	Absent		PE/TCE	2
5	333	Present	Blood, hair	Present	Chitin	Absent		PE/TCE	2
4	439	Absent		Present	Chitin	Absent		PE/TCE	1
2	447	Absent		Present	Chitin	Absent		PE/TCE	1
6	479	Present	Bone	Present	Chitin	Absent		PE/TCE	2
3	545	Present	Plasma	Present	chitin	Absent		PE/TCE	2

The plasma (if it was indeed plasma, we are honestly not entirely sure) partices found in 545 were highly unusual and in very low concentrations. They seemed inhomogeneously distributed throuhout the sample. With some slides containing a decent amount of them and others containing none at all.

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.	Fractions used	Number of determinations
1	109	Present	bones, muscle fibres	Absent		Absent		PE/TCE	2
2	219	Absent		Present	muscle fibres, cuticula, tracheal system	Absent		PE/TCE	1
4	355	Absent		Absent		Absent		PE/TCE	1
6	371	Present	bones	Absent		Absent		PE/TCE	2
5	441	Present	bones, hair, blood, cartilage	Absent		Absent		PE/TCE	2
3	497	Absent		Absent		Absent		PE/TCE	1

441: nearly all the blood and hair particles were found in the final flotate

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.	Fractions used	Number of determinations
1	37	Present	bones, cartilages, blood particles and muscle fibers.	Absent		Absent		PE/TCE	2
2	63	Absent		Present	cuticules, muscle fibers.	Absent		PE/TCE	2
6	155	Present	bones, cartilages.	Absent		Absent		PE/TCE	2
5	321	Present	blood, hair and 2 little fragments of muscle fibers.	Absent		Absent		PE/TCE	2
3	509	Absent		Absent		Absent		PE/TCE	2
4	595	Absent		Absent		Absent		PE/TCE	2

Sample 509: In this sample two particles were seen that appears to be from invertebrat but not with sufficient characteristics for a clear identification. We also saw in this sample low presence of muscle fibers fragments, which cannot be categorised as terrestrial vertebrates, fish or invertebrates.

Sample 155: In this sample one particle was seen which could be from invertebrate, but not with sufficient characteristics for a clear identification.

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.	Fractions used	Number of determinations
1	169	Present	bones	Absent		Absent		PE/TCE	2
3	197	Absent		Absent		Absent		PE/TCE	1
6	203	Present	bones	Absent		Absent		PE/TCE	2
4	271	Absent		Absent		Absent		PE/TCE	1
2	339	Absent		Present	insect cuticles	Absent		PE/TCE	2
5	657	Present	hairs and blood meal	< LOD	'insect cuticles	Absent		PE/TCE	2

'in the sample No 339 was observed microscopic mites. In the sample No 169 was detected >10 particle of muscles in flotate after PE/TCE. In the sample No 203 was detected 5 particles of muscles in flotate after PE/TCE.

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.	Fractions used	Number of determinations
1	157	Present	Bones, cartilage, muscles	Absent		Absent		PE/TCE	1
6	347	Present	bones	Absent		Absent		PE/TCE	1
4	367	Absent		Present	insect particles	Absent		PE/TCE	1
2	411	Absent		Present	insect particles	Absent		PE/TCE	1
5	585	Present	blood particles, hairs	Present	insect particles	Absent		PE/TCE	1
3	641	Absent		Absent		Absent		PE/TCE	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.	Fractions used	Number of determinations
6	119	Present	bones	Absent		Absent		PE/TCE	1
2	123	Absent		Present	cuticles, muscle fibers	Absent		PE/TCE	1
5	225	Present	hairs, blood	Absent		Absent		PE/TCE	1
3	485	Absent		Absent		Absent		PE/TCE	1
1	505	Present	bones, plasma suspicion	Absent		Absent		PE/TCE	1
4	655	Absent		Absent		Absent		PE/TCE	1

Sample 225 - TMB test : Colored particles in blue and presence of bubbles

Sample 505 - TMB test : colored particles in blue and no bubbles

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.	Fractions used	Number of determinations
6	23	Present	bones	< LOD	insect	Absent		PE/TCE	2
2	111	Absent		Present	insect	Absent		PE/TCE	2
3	317	Absent		< LOD	insect	Absent		PE/TCE	2
1	529	Present	bones	Absent		Absent		PE/TCE	2
5	549	Present	blood, hair	Absent		Absent		PE/TCE	2
4	643	Absent		Absent		Absent		PE/TCE	1

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.	Fractions used	Number of determinations
1	133	Present	Bones	Absent		Absent		PE/TCE	1
6	179	Present	Bones	Absent		Absent		PE/TCE	1
3	209	Absent		Absent		Absent		PE/TCE	1
2	435	Absent		Present	Cuticule	Absent		PE/TCE	1
4	463	Absent		Present	Cutice	Absent		PE/TCE	1
5	489	Present	Hemoglobin, hair	Absent		Absent		PE/TCE	1

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.	Fractions used	Number of determinations
3	65	Absent		Present	cuticular fragments	Absent		PE/TCE	2
1	181	Present	bones, muscles	Absent		Absent		PE/TCE	2
2	231	Absent		Present	cuticular fragments, muscles	Absent		PE/TCE	2
6	443	Present	bones	Absent		Absent		PE/TCE	2
4	547	Absent		Absent		Absent		PE/TCE	2
5	645	Present	bones, hairs	Absent		Absent		PE/TCE	2

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.	Fractions used	Number of determinations
1	61	Present	bones	Absent		Present		PE/TCE	1
3	293	Absent		Absent		Absent		PE/TCE	1
2	375	Absent		Present	cuticula	Absent		PE/TCE	1
6	395	Present	bones	Absent		Absent		PE/TCE	1
5	597	Present	blood, hear	Absent		Absent		PE/TCE	1
4	619	Absent		Absent		Absent		PE/TCE	1

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.	Fractions used	Number of determinations
5	129	Present	hair	Absent		Absent		PE/TCE	1
3	137	Absent		Absent		Absent		PE/TCE	1
4	187	Absent		Absent		Absent		PE/TCE	1
2	399	Absent		Present	mucels, parts of insects	Absent		PE/TCE	1
1	553	Present	bones, cartilage	Absent		Absent		PE/TCE	1
6	623	Present	bones, cartilage	Absent		Absent		PE/TCE	1

Raw material, sediment 1 + 2 and flotate has been examined in all samples

Samples has been examined for the precence of blood, plasma and milk

Glycerol, Norland 65, 2,5% NaOH, Fehlings solution and iodine has been used for determination

Sediment 1 has been stained with Alizarin

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.	Fractions used	Number of determinations
6	263	Present	bones, muscles	Absent		Absent		PE/TCE	
5	285	Present	hair, blood	Present	whole insect, cuticule	Absent		PE/TCE	
2	291	Absent		Present	cuticule	Absent		PE/TCE	
4	331	Absent		Present	cuticule	Present	scales	PE/TCE	
3	353	Absent		Present	cuticule	Absent		PE/TCE	
1	649	Present	bones, muscles	Absent		Absent		PE/TCE	

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.	Fractions used	Number of determinations
6	107	Present	terrestrial vertebrates bones			Absent		TCE	2
3	125	Absent		Present	cuticle fragments, insect appendages	Absent		PE/TCE	1
5	141	Present	hairs, blood			Absent		TCE	2
1	517	Present	terrestrial vertebrates bones			Absent		TCE	2
2	615	Absent		Present	cuticle fragments, tracheoles structures, claws/pinchers/other appendages	Absent		PE/TCE	1
4	631	Absent		Present	cuticle fragments, insect appendages	Absent		PE/TCE	1

Samples 125, 631: The number of insect particles that we could confidently identify was just slightly above the LOD (6 to 8 particles in each of these samples). Therefore, samples were considered positive for terrestrial invertebrates after 1 determination (>5 particles).
Sample 141: raw material stained with TMB+H2O2, blue colour development within seconds, and appearance of air bubbles. We noticed the presence of insect particles, but the double sedimentation was not applied in accordance to the EURL-SOP "Operational Schemes" (presence of blood products and hairs). We also noticed the presence of particles vaguely resembling ossicles in the sediment. However, we could't unambigously identify them as ossicles.

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.	Fractions used	Number of determinations
5	21	Present	Bood, hair	Absent		Absent		PE/TCE	2
3	185	Absent		Present		Absent		PE/TCE	2
4	307	Absent		Absent		Absent		PE/TCE	2
6	491	Present	Bones	Absent		Absent		PE/TCE	2
2	591	Absent		Present		Absent		PE/TCE	2
1	601	Present	Bones	Absent		Absent		PE/TCE	2

Laboratory identification code : **25**

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.	Fractions used	Number of determinations
4	19	Absent		Absent		Absent		PE/TCE	1
5	105	Present	hairs, blood meal	Absent		Absent		PE/TCE	1
2	363	Present	bones, muscle fibres it can't be exluded muscle fibres found only derive from terr. vertebrates and terr. invertebrates	Present	cuticula, muscle fibres it can't be exluded muscle fibres found only derive from terr. vertebrates and terr. invertebrates	Absent		PE/TCE	2
3	377	Absent		< LOD	in total 4 particles of cuticula, 1 muscle fibre it can't be exluded muscle fibres found only derive and terr. invertebrates	Absent		PE/TCE	2
1	493	Present	bones, muscle fibres it can't be exluded muscle fibres found only derive from terrestrial vertebrates	Absent		Absent		PE/TCE	1
6	599	Present	bones, muscle fibres it can't be exluded muscle fibres found only derive from terrestrial vertebrates	Absent		Absent		PE/TCE	1

Sample 363: insect meal may come from tenebrio molitor, Sample 377: particles of insect described may be a contamination

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.	Fractions used	Number of determinations
4	31	Absent		Absent		Absent		PE/TCE	2
3	113	Absent		Absent		Absent		PE/TCE	2
1	397	Present	Bones	Absent		Absent		PE/TCE	2
5	405	Present	Blood (blood meal). Hair	Absent		Absent		PE/TCE	2
2	627	Absent		Present	Cuticule, muscle fibers.	Absent		PE/TCE	2
6	635	Present	Bones	Absent		Absent		PE/TCE	2

For Samples 31 and 113 (1st and 2nd), two determinations were carried-out, although it would not have been necessary according to the legal protocol. This was done because several people are qualified for this type of analysis in the lab and the entire procedure was performed twice by 2 different people as an exercise.

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.	Fractions used	Number of determinations
4	79	Absent		Absent		Absent		TCE	2
3	149	Absent		Absent		Present	presence of fish bone fragments	TCE	2
5	249	Present	presence of hairs	Absent		Present	presence of fish bone fragments	TCE	2
1	373	< LOD	presence of terrestrial bone	Absent		Absent		TCE	2
6	419	Present	presence of terrestrial bone	Absent		Absent		TCE	2
2	459	Absent		Absent		Absent		TCE	2

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.	Fractions used	Number of determinations
2	99	Absent		Absent		Absent		TCE	1
5	189	Absent		Present	Bones	Absent		TCE	1
6	311	Present	Bones	Present	Bones	Absent		TCE	1
1	613	Present	Bones	Absent		Absent		TCE	1
3	629	Present	bones	Present	bones	Absent		TCE	1
4	7	< LOD	bones	Present	bones	Absent		TCE	1

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.	Fractions used	Number of determinations
2	15	Absent		Absent		Absent		PE/TCE	1
3	53	Absent		Absent		Absent		PE/TCE	1
6	239	< LOD	5 particles of bone	Absent		Absent		PE/TCE	1
1	421	Present	positive for terrestrial bone	Absent		Absent		PE/TCE	1
5	429	Present	Positive for blood (TMB, raw material)	Absent		Present	bone+scale	PE/TCE	1
4	571	Absent		Absent		Present	bone+scale	PE/TCE	1

Laboratory identification code : **30**

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.	Fractions used	Number of determinations
5	9	Present	Blood, hair	Absent		Absent		PE/TCE	2
2	51	Absent		Present	Cuticule	Absent		PE/TCE	2
3	569	Absent		Absent		Absent		PE/TCE	2
4	583	Absent		Absent		Absent		PE/TCE	2
6	587	Present	Bones	Absent		Absent		PE/TCE	2
1	589	Present	Bones	Absent		Absent		PE/TCE	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.	Fractions used	Number of determinations
1	13	Present	bone and muscle fibres.			Absent		TCE	1
5	273	Present	blood and hair			Absent		TCE	1
3	281	Absent		Present		Absent		PE/TCE	2
4	343	Absent		< LOD		Absent		PE/TCE	2
6	359	Present	bone and mucle fibres			Absent		TCE	1
2	603	Absent		Present		Absent		PE/TCE	2

Laboratory identification code : **34**

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.	Fractions used	Number of determinations
2	39	Absent		Present	Insect fragments, cuticles	Present	Muscle fibres, fishbone	Chloro	2
1	85	Absent		Absent		Present	Fishbone	Chloro	2
5	153	Present	Hairs, Blood	Absent		Absent		Chloro	2
3	233	Absent		Absent		Absent		Chloro	2
4	403	Absent		Present	Insect fragments, mouthparts	Absent		Chloro	2
6	659	Absent		Present	Insect fragments, cuticles	Present	Muscle fibres, fishbone	Chloro	2

Annex 5

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

Laboratory identification code : **1**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,45	38,15	37,74	cycles
Copy number at the cut-off :	11,64	9,79	3,66	copies
Master mix used :	Universal master mix, Diagenode			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
6	**47**	Present	Absent	Absent	
3	**173**	Absent	Absent	Absent	
1	**193**	Present	Absent	Absent	
2	**351**	Absent	Absent	Absent	
5	**465**	Absent	Present	Absent	
4	**559**	Absent	Absent	Absent	

Laboratory identification code : **2**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,77	38,87	40,90	cycles
Copy number at the cut-off :	9,56	9,48	3,13	copies
Master mix used :	DMMLD2D100 (GMO-UN-600, RT-QP2X-03			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
2	**3**	Absent	Absent	Absent	
1	**49**	Present	Absent	Absent	
6	**59**	Present	Absent	Absent	
3	**77**	Absent	Absent	Absent	
4	**475**	Absent	Absent	Absent	
5	**525**	Absent	Present	Absent	

Laboratory identification code : **3**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	34,23	36,59	37,86	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	Pig DNA	Poultry DNA	Comment
3	**5**	Absent	Absent	Absent	
6	**83**	Present	Absent	Absent	
2	**159**	Absent	Absent	Absent	During extraction magnetic beads clump together and was difficult to resuspend
1	**229**	Present	Absent	Absent	
5	**369**	Absent	Present	Absent	During extraction magnetic beads clump together and was difficult to resuspend
4	**451**	Absent	Absent	Absent	During extraction magnetic beads clump together and was difficult to resuspend

Laboratory identification code : **4**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	34,98	40,49	38,30	cycles
Copy number at the cut-off :	9,00	7,92	3,32	copies
Master mix used :	Diagnode Universal mastermix GMO-UN-A600			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
2	**87**	Absent	Absent	Absent	Pig negative according to standard curve on plate
6	**167**	Present	Absent	Absent	Pig negative according to standard curve on plate
1	**241**	Present	Absent	Absent	
4	**427**	Absent	Absent	Absent	
3	**461**	Absent	Absent	Absent	
5	**513**	Absent	Present	Absent	

Laboratory identification code : **5**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	33,51	35,93	40,20	cycles
Copy number at the cut-off :	10,85	9,25	3,07	copies
Master mix used :	Diagenode Universal Mastermix			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
6	**95**	Present	Absent	Absent	
1	**217**	Present	Absent	Absent	
2	**243**	Absent	Absent	Absent	
3	**449**	Absent	Absent	Absent	
4	**523**	Absent	Absent	Absent	
5	**609**	Absent	Present	Absent	

Laboratory identification code : **6**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,48	38,85	38,05	cycles
Copy number at the cut-off :	10,56	10,92	3,32	copies
Master mix used :	Brilliant II QPCR Low ROX Master Mix, cat. N. 600806			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
6	**35**	Present	Absent	Absent	
1	**97**	Present	Absent	Absent	
4	**115**	Absent	Absent	Absent	
5	**177**	Absent	Present	Absent	
2	**315**	Absent	Absent	Absent	
3	**437**	Absent	Absent	Absent	

Laboratory identification code : **7**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,30	38,32	39,33	cycles
Copy number at the cut-off :	11,00	9,64	3,76	copies
Master mix used :		Eurogentec		

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
4	**175**	Absent	Absent	Absent	
1	**205**	Present	Absent	Absent	
2	**267**	Absent	Absent	Absent	
6	**539**	Present	Absent	Absent	
5	**561**	Absent	Present	Absent	
3	**617**	Absent	Absent	Absent	

Laboratory identification code : **8**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,20	41,37	39,51	cycles
Copy number at the cut-off :	11,48	3,59	9,06	copies
Master mix used :		EUROGENTEC		

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
2	**147**	Absent	Absent	Absent	Poultry: partial inhibition
6	**299**	Present	Absent	Absent	Poultry: partial inhibition
3	**305**	Absent	Absent	Absent	Poultry: partial inhibition
1	**445**	Present	Absent	Absent	Poultry: partial inhibition
5	**453**	Present	Present	Absent	Poultry: partial inhibition
4	**607**	Present	Absent	Absent	Poultry: partial inhibition

Laboratory identification code : **9**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,22	37,39	38,91	cycles
Copy number at the cut-off :	10,53	11,45	3,58	copies
Master mix used :		Brilliant II QPCR Low Rox Master Mix		

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
2	**27**	Absent	Absent	Absent	
3	**89**	Absent	Absent	Absent	
1	**121**	Present	Absent	Absent	
6	**335**	Present	Absent	Absent	
4	**499**	Absent	Absent	Absent	
5	**501**	Absent	Present	Absent	

Laboratory identification code : **10**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,75	39,89	41,01	cycles
Copy number at the cut-off :	11,97	9,46	3,27	copies
Master mix used :		Eurogentec RT-QP2X-03		

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	**25**	Present	Absent	Absent	
6	**131**	Present	Absent	Absent	
5	**381**	Absent	Present	Absent	
3	**413**	Absent	Absent	Absent	
4	**415**	Absent	Absent	Absent	
2	**507**	Absent	Absent	Absent	

Laboratory identification code : **11**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,18	37,36	37,67	cycles
Copy number at the cut-off :	11,84	10,27	3,50	copies
Master mix used :		Eurogentec RT-QP2X-03NR		

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	**1**	Present	Absent	Absent	
5	**333**	Absent	Present	Absent	
4	**439**	Absent	Absent	Absent	
2	**447**	Absent	Absent	Absent	
6	**479**	Present	Absent	Absent	
3	**545**	Absent	Absent	Absent	

Laboratory identification code : **12**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,45	36,13	37,07	cycles
Copy number at the cut-off :	9,29	11,33	3,83	copies
Master mix used :		Agilent Brilliant II, low Rox		

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	**109**	Present	Absent	Absent	
2	**219**	Absent	Absent	Absent	
4	**355**	Absent	Absent	Absent	
6	**371**	Present	Absent	Absent	
5	**441**	Absent	Present	Absent	
3	**497**	Absent	Absent	Absent	

Laboratory identification code : **13**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	37,84	38,00	38,97	cycles
Copy number at the cut-off :	9,37	9,60	3,45	copies
Master mix used :	Diagenode DMML-D2-D600			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	**37**	Present	Absent	Absent	All the fortified samples had Ct results confirming that no inhinition occured.
2	**63**	Absent	Absent	Absent	All the fortified samples had Ct results confirming that no inhinition occured.
4	**155**	Present	Absent	Absent	All the fortified samples had Ct results confirming that no inhinition occured.
6	**321**	Absent	Present	Absent	All the fortified samples had Ct results confirming that no inhinition occured.
5	**509**	Absent	Absent	Absent	All the fortified samples had Ct results confirming that no inhinition occured.
3	**595**	Absent	Absent	Absent	All the fortified samples had Ct results confirming that no inhinition occured.

Laboratory identification code : **14**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	34,66	39,32	41,35	cycles
Copy number at the cut-off :	10,17	9,57	3,12	copies
Master mix used :	TaqMan Universal Master Mix AB			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	**169**	Present	Absent	Absent	
3	**197**	Absent	Absent	Absent	
6	**203**	Present	Absent	Absent	
4	**271**	Absent	Absent	Absent	
2	**339**	Absent	Absent	Absent	'PCR inhibition
5	**657**	Absent	Present	Absent	PCR inhibition

Laboratory identification code : **15**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,66	36,87	38,94	cycles
Copy number at the cut-off :	11,29	11,63	3,99	copies
Master mix used :	Brilliant II QPCR Low ROX Master mix			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	**157**	Present	Absent	Absent	
6	**347**	Present	Absent	Absent	
4	**367**	Absent	Absent	Absent	
2	**411**	Absent	Absent	Absent	
5	**585**	Absent	Present	Absent	
3	**641**	Absent	Absent	Absent	

Laboratory identification code : **16**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	37,26	41,65	40,97	cycles
Copy number at the cut-off :	11,14	11,02	3,30	copies
Master mix used :		AGILENT - Ref : 600806		

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
6	**119**	Present	Absent	Absent	
2	**123**	Absent	Absent	Absent	
5	**225**	Absent	Present	Absent	
3	**485**	Absent	Absent	Absent	
1	**505**	Present	Absent	Absent	
4	**655**	Absent	Absent	Absent	

Laboratory identification code : **17**

	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	Pig DNA	Poultry DNA	Comment
6	**23**	Present	Absent	Absent	
2	**111**	Absent	Absent	Absent	
3	**317**	Absent	Absent	Absent	
1	**529**	Present	Absent	Absent	
5	**549**	Absent	Present	Absent	
4	**643**	Absent	Absent	Absent	

Laboratory identification code : **18**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,74	36,36	36,31	cycles
Copy number at the cut-off :	10,17	9,11	3,10	copies
Master mix used :	Universal Master Mix DDML-D2-D600 Expire:12/2023			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
6	**133**	Present	Absent	Absent	PCR total inhibition for poultry DNA (Tested with Level 3calibrant) Tested with dilutions x20,x30,x40 for porcine dna with negative results No total inhibition in porcine DNA
2	**179**	Present	Absent	Absent	PCR total inhibition for poultry DNA (Tested with Level 3calibrant) Tested with dilutions x20,x30,x40 for porcine dna with negative results No total inhibition in porcine DNA
3	**209**	Absent	Absent	Absent	PCR total inhibition for poultry DNA (Tested with Level 3calibrant) Tested with dilutions x20,x30,x40 for porcine dna with negative results No total inhibition in porcine DNA
1	**435**	Absent	Absent	Absent	PCR total inhibition for poultry DNA (Tested with Level 3calibrant) Tested with dilutions x20,x30,x40 for porcine dna with negative results No total inhibition in porcine DNA
5	**463**	Absent	Absent	Absent	PCR total inhibition for poultry DNA (Tested with Level 3calibrant) Tested with dilutions x20,x30,x40 for porcine dna with negative results No total inhibition in porcine DNA
4	**489**	Absent	Present	Absent	PCR total inhibition for poultry DNA (Tested with Level 3calibrant)

Laboratory identification code : **19**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,04	38,84	39,13	cycles
Copy number at the cut-off :	12,47	11,00	4,58	copies
Master mix used :				

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
3	65	Absent	Absent	Absent	
1	181	Present	Absent	Absent	
3	231	Absent	Absent	Absent	
6	443	Present	Absent	Absent	
4	547	Absent	Absent	Absent	
5	645	Absent	Present	Absent	

Laboratory identification code : **20**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	30,62	9,09	39,32	cycles
Copy number at the cut-off :	9,16	10,78	3,58	copies
Master mix used :	Eurogenetec, qPCR 2X MasterMix			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	61	Absent	Absent	Absent	White precipitate in DNA extraction Spiked 1 fold samples obtained positive results
3	293	Absent	Absent	Absent	White precipitate in DNA extraction Spiked 1 fold samples obtained positive results
2	375	Absent	Absent	Absent	White precipitate in DNA extraction Spiked 1 fold samples obtained positive results
6	395	Absent	Absent	Absent	White precipitate in DNA extraction Spiked 1 fold samples obtained positive results
5	597	Absent	Present	Absent	White precipitate in DNA extraction Spiked 1 fold samples obtained positive results
4	619	Absent	Absent	Absent	White precipitate in DNA extraction Spiked 1 fold samples obtained positive results

Laboratory identification code : **21**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,24	37,28	38,69	cycles
Copy number at the cut-off :	10,36	9,06	3,05	copies
Master mix used :	Universal mastermix, Diagenode			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
5	129	Absent	Present	Absent	
3	137	Absent	Absent	Absent	
4	187	Absent	Absent	Absent	
2	399	Absent	Absent	Absent	
1	553	Present	Absent	Absent	
6	623	Present	Absent	Absent	

Laboratory identification code : **22**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	32,21	35,83	34,18	cycles
Copy number at the cut-off :	12,81	10,93	3,42	copies
Master mix used :	bioline sensifast probe no-rox kit			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
6	**263**	Present	Absent	Absent	
5	**285**	Absent	Present	Absent	
2	**291**	Absent	Absent	Absent	
4	**331**	Absent	Absent	Absent	
3	**353**	Absent	Absent	Absent	
1	**649**	Present	Absent	Absent	

Laboratory identification code : **23**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,46	35,92	37,67	cycles
Copy number at the cut-off :	11,84	11,96	3,89	copies
Master mix used :	Ruminant : Taqman Universal PCR Master Mix (Thermofisher, product code 4304437);			
Master mix used :	Poultry and Pig : Brilliant II QPCR Low ROX Master Mix (Agilent, product code 600806)			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
6	**107**	Present	Absent	Absent	
3	**125**	Absent	Absent	Absent	
5	**141**	Absent	Present	Absent	
1	**517**	Present	Absent	Absent	
2	**615**	Absent	Absent	Absent	
4	**631**	Absent	Absent	Absent	

Laboratory identification code : **24**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	34,44	37,31	39,32	cycles
Copy number at the cut-off :	10,66	9,26	3,23	copies
Master mix used :	Universal mastermix Diagenode (Exp.31/12/2023)			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
5	**21**	Absent	Present	Absent	
3	**185**	Absent	Absent	Absent	
4	**307**	Absent	Absent	Absent	
6	**491**	Present	Absent	Absent	
2	**591**	Absent	Absent	Absent	
1	**601**	Present	Absent	Absent	

Laboratory identification code : **27**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	30,00	30,00	35,00	cycles
Copy number at the cut-off :				copies
Master mix used :	ſec Probe PCR kit - For quantitative, real-time PCR and tv			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
4	**79**	Absent	Absent	Absent	The samples were analyzed in a simplified manner, using a Roche LightCycler 2,0 thermal cycler
3	**149**	Absent	Present	Absent	
5	**249**	Absent	Present	Absent	
1	**373**	Absent	Absent	Absent	
6	**419**	Absent	Absent	Absent	
2	**459**	Absent	Absent	Absent	

Laboratory identification code : **29**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	37,87	37,17	39,52	cycles
Copy number at the cut-off :	11,51	11,06	3,98	copies
Master mix used :	Brilliant II QPCR Master Mix With Low ROX (Agilent)			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
2	**15**	Absent	Absent	Absent	
3	**53**	Absent	Absent	Absent	
6	**239**	Present	Absent	Absent	
1	**421**	Present	Absent	Absent	
5	**429**	Absent	Present	Absent	
4	**571**	Absent	Absent	Absent	

Laboratory identification code : **30**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,54	37,06	38,86	cycles
Copy number at the cut-off :	10,32	10,23	3,83	copies
Master mix used :	Agilent Brilliant II QPCR 600806			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
5	**9**	Absent	Present	Absent	
2	**51**	Absent	Absent	Absent	
3	**569**	Absent	Absent	Absent	
4	**583**	Absent	Absent	Absent	
6	**587**	Present	Absent	Absent	
1	**589**	Present	Absent	Absent	

Laboratory identification code : **32**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,29	37,30	36,08	cycles
Copy number at the cut-off :	10,34	0,00	0,00	copies
Master mix used :	Eurogentec - qPCR 2X MasterMix Plus 7.5ml			

Sample type	Sample N°	Ruminant DNA	Pig DNA	Poultry DNA	Comment
1	**13**	Present	Absent	Absent	Porcine and Avian PCR results are from an In House PCR. Weak avian signal seen beyond cut-off.
5	**273**	Absent	Absent	Absent	Porcine and Avian PCR results are from an In House PCR. Weak avian signal seen beyond cut-off. Weak Porcine signal seen beyond cut off.
3	**281**	Absent	Absent	Absent	Porcine and Avian PCR results are from an In House PCR
4	**343**	Absent	Absent	Absent	Porcine and Avian PCR results are from an In House PCR
6	**359**	Present	Absent	Absent	Porcine and Avian PCR results are from an In House PCR
2	**603**	Absent	Absent	Absent	Porcine and Avian PCR results are from an In House PCR