Combined microscopy-PCR
EURL-AP Proficiency Test 2024

Final version

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Summary

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

The total number of participating laboratories was 29 (26 NRLs and 3 labs outside the NRL network). The study was based on a set of five samples (to be analysed both by light microscopy and PCR) consisting of blank feed matrices or feed materials fortified or not with processed animal proteins from terrestrial vertebrates, terrestrial invertebrates and/or from fish.

Results from light microscopy showed that the number of NRLs obtaining an excellent score was of 73 %, that of obtaining satisfactory scores was of 23 %. Only one NRL was underperforming. The design of sample set allowed to demonstrate that in presence of salmon meal the risk of false positives for terrestrial vertebrates was a concern; six results erroneously reported the presence of terrestrial bones in such situations. This type of specificity issue is however reported over the past and is illustrating the necessity of maintaining an expertise to avoid such errors.

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-six % of the NRLs (25 out of 26) performed excellently and reported no false result. One NRL (4 %) was considered as satisfying: one positive deviation was recorded for the detection of poultry DNA. The global performance of the network remained excellent with the three PCR methods (with only one deviation out of the 364 PCR results recorded the global accuracy of the network is 0.997).

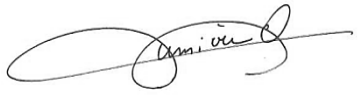
Keywords :

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 24th February 2025. After reception and evaluation of the comments on the 11th March 2025, it was amended accordingly and approved by the signature of the organisers.

ISO 17043 coordinators signature for approval:

Olivier Fumière *Pascal Veys*



1. Foreword

European Union Reference Laboratories (EURLs) were created 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 (SOPs) are supporting the implementation of the two methods.

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

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

3. Material and methods

3.1. Study organisation

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

Official announcement (Annex 1) of the study was made on the 6th September 2024 to all invited participants.

On the 31st October 2024, the sample sets were shipped to the participants. On that same day the Excel report forms containing the instructions (Annex 2) 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 29th November 2024.

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 2). 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.

On the exception of one non-EU participant, all results were delivered on time to the organiser. Thus, the study presents results from 29 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. The composition of the sample set was established considering the following factors:

- Use of feed and feed materials intended to pigs, poultry and fish ;
- Use of pure ingredients from animal origin commercially available ;
- Use of milk containing compound feed hardly detectable by light microscopy but generating a positive ruminant signal by PCR ;
- Use of insect material ;
- Use of a salmon fishmeal presenting peculiar bone lacunae.

Each participating lab received thus a sample set of five 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	Pigfeed + 0.1% feather meal + 1% egg	+	-	-	-	-	+
2	Pigfeed (containing milk) + 1% salmon	-	-	+	+	-	-
3	Poultry feed + 0.5% *T. molitor*	-	+	-	n.a.	-	-
4	Pigfeed (blank)	-	-	-	-	-	-
5	Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder	+	-	+	+	+	-
Total positive results		**2**	**1**	**2**	**2**	**1**	**1**

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

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

An error was noticed in the sent tabulation results forms: sample 2 was erroneously typed intended positive for terrestrial vertebrates' presence instead of negative. This error had no influence on the proficiency assessment.

3.2.2. Materials used in the preparation of the samples

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

- A first **pig feed**, used to prepare sample 1 and sample 4 was a commercial fattening compound feed. Its composition was unknown. Its sediment content was of about 1 %. No animal remains could be detected by microscopy. No animal DNA was detected by PCR.
- A second **pig feed,** used in sample 2, was a commercial compound feed for weaning piglets. It was containing dairy product (as detected by mass spectrometry and PCR). Its sediment was of 2 %. The feed was positive for ruminant DNA presence.
- The **poultry feed**, used in sample 3 was a complete feed meat chicken. It was composed of maize, wheat, unhulled soja seeds, wheat bran, rapeseed, peas, calcium carbonate and soja oil. Its sediment was of 2.5 %. No DNA from animal origin was detected by PCR.
- The last feed, **a fishfeed**, used for preparing sample 5, was a complement pelleted feed salmon. Its composition was made of soja protein concentrate, plant and fish oils, fishmeal, horse bean, wheat gluten, corn gluten meal, wheat and minerals. Its sediment was of 1 %. No other animal DNA (ruminant, pig or poultry DNA) than fish DNA was detected by PCR.

Adulterant material used:

- A **poultry feather meal** was used for preparing sample 1. It had no sediment. Only poultry DNA was detected by PCR.
- An **egg powder**, intended for human consumption was used for preparing sample 1. It was free from sediment. PCR analyses revealed only the presence of poultry DNA.
- A pure **salmon meal** was used for sample 2. Its sediment was 15 %. Only DNA from fish was detected by PCR.
- An **insect meal** was used for preparing sample 3. It was produced from the industry and consisted only of *Tenebrio molitor*, the mealworm. No traces of DNA from the animal species tested (ruminant, pig and poultry) could be detected by PCR.
- A **bovine PAP** was used to prepare sample 5. Its sediment content was of 60 %. PCR analyses revealed only the presence of ruminant DNA.

- A **porcine haemoglobin powder** was also used for sample 5. It had no sediment. Only porcine DNA was detected by PCR.

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.

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

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

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

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

3.4. Performance criteria

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

3.4.1. Light microscopy

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

Concerning the presence of terrestrial vertebrates:

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

Concerning the presence of terrestrial invertebrates:

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

Concerning the presence of fish:

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

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

- **Excellent** level of global performance: consolidated AC = 1.00 or faultless set of results.
- **Satisfying** level of global performance: consolidated AC ≥ 0.86 without ND for terrestrial vertebrates and terrestrial invertebrates.
- **Underperforming** level of global performance: consolidated AC < 0.86 OR ≥ 0.86 with one ND for terrestrial vertebrates.

3.4.2. PCR

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

- Sample 1 was considered to be positive for the presence of poultry DNA.
- Sample 2 and 5 were considered to be declared positive for the presence of ruminant DNA.
- Sample 4 had to be declared negative.
- Sample 5 was considered to be positive for the presence of pig DNA.

The detection of ruminant DNA for sample 3 was out of the performance assessment.

Concerning the PCR, the performance criteria were decided as:

- **Excellent** level of global performance: global AC = 1.00 with no false result (ND or PD) for the detection of ruminant, pig and poultry DNA.
- **Satisfying** level of global performance: global AC ≥ 0.93 with maximum 1 false result (ND or PD) for the detection of pig and poultry DNA and no deviation for the detection of ruminant DNA.
- **Underperforming** level of global performance: global AC ≥ 0.93 with 1 false result (ND or PD) for the detection of ruminant DNA or global AC < 0.93 with 2 false results (ND or PD) or more.

3.5. Homogeneity study

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

Table 2: Homogeneity study – Results

Sample	Material		Light microscopy			PCR			
		Nr of replicates	Terrestrial vert.	Terrestrial invert.	Fish	Nr of replicates	Ruminant	Porcine	Poultry
1	Pigfeed + 0.1% feather meal + 1% egg	10	+	-	-	10	-	+	+
2	Pigfeed (containing milk) + 1% salmon	10	-	-	+	10	+	-	-
3	Poultry feed + 0.5% *T. molitor*	10	-	+	-	10	*	-	-
4	Pigfeed (blank)	10	-	-	-	10	-	-	-
5	Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder	10	+	-	+	10	+	+	-

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

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

Sample 1 (Pigfeed + 0.1% feather meal + 1% egg) was systematically positive for the presence terrestrial vertebrates with the observations feathers fragments and few bone fragments (7 replicates with ≤ 5 bones). No traces of terrestrial invertebrates were detected. PCR analyses detected systematically the presence of poultry DNA whereas ruminant and pig were absent.

Sample 2 (Pigfeed (containing milk) + 1% salmon) was free from any terrestrial vertebrate or invertebrates' particles. The finding of fishbones and muscle fibres was systematic. Ruminant DNA was systematically detected without any detectable traces of pig and poultry DNA.

Sample 3 (Poultry F + 0.5% *T. molitor*) was free from any terrestrial vertebrates and fish presence. All replicates were positive for terrestrial invertebrates' fragments. No DNA from pig and poultry was detected by PCR analyses on the items used for the homogeneity study. The presence of ruminant DNA was sporadically detected (1 positive and 4 ambiguous items out of the 10 tested). For that reason, this parameter was kept out of the evaluation.

Sample 4 (Pigfeed) was free from any animal presence (on the exception of the finding of one mite through the whole series of 10 replicates). No DNA from ruminant, pig and poultry was detected by PCR analyses.

Sample 5 (Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder) was positive for the presence of fish and terrestrial vertebrates. Among the terrestrial vertebrates' findings, the presence of bones, muscle fibres and haemoglobin globules were systematically recorded. No trace of invertebrates could be found. Only DNA from ruminant and pig were detected by PCR analyses.

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.

In addition, this year, one of the ten replicates of each material used in the homogeneity study was analysed by PCR after the results submission deadline. All results obtained from this second analysis aligned with expectations, confirming the stability of the samples

4. Results

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

4.1. Microscopy results

4.1.1. Qualitative analyses from the NRLs

4.1.1.1. Results and performance of the network

Table 3 summarizes the results reported by the 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 only 2 % of the total responses.

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

Sample	Material	n	AC		
			Terr. Vert.	Terr. Inv.	Fish
1	Pigfeed + 0.1% feather meal + 1% egg	26	1.000	0.962 (1)	1.000
2	Pigfeed (containing milk) + 1% salmon	26	0.769 (6)	1.000	1.000
3	Poultry feed + 0.5% *T. molitor*	26	1.000	1.000	1.000
4	Pigfeed (blank)	26	1.000	0.923 (2)	1.000
5	Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder	26	1.000	1.000	1.000

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

Regarding the detection of terrestrial vertebrates' constituents, the sensitivity was perfect without any false negative results. The only issue dealt with the specificity. Six false positive cases were reported for sample 2, which represents two thirds of the total errors.

The detection of terrestrial invertebrates demonstrated also perfect sensitivity score for insect particles. Problems of specificity were noted for sample 1 and 4. They represented a third of the total errors.

The detection of fish constituents occurred faultless both for the sensitivity and the specificity.

Further details on the errors noted in this study are in described in next point. Possible explanations for the problems are presented in the discussion section.

4.1.1.2. Detailed review of results per sample

Sample 1: Pigfeed + 0.1% feather meal + 1% egg

All results were correct for the terrestrial vertebrate parameter. However, the presence of feather fragments was only reported by 16 NRLs (62 %). None of the NRLs disclosed the presence of egg powder which was not a surprise since this type of material is uncommon.

PD for terrestrial invertebrates' particles:

- Lab 24 reported cuticle fragments, muscles and tracheal structures

All results were correct for the fish parameter

Sample 2: Pigfeed (containing milk) + 1% salmon

PD for terrestrial vertebrates' particles:

- Labs 3, 6, 10, 11, 18 and 24 reported the presence of bones.

Few bone fragments, at levels < LOD were also reported by labs 1, 7, 23 and 26.

In addition to these observations, seven NRLs (27 %) classified this sample as positive for terrestrial vertebrates but according to the finding of dairy particles solely. Since the pigfeed was known to contain milk, there results were considered as correct.

All results were correct for the terrestrial invertebrates and fish parameters

Samples 3: Poultry feed + 0.5% *T. molitor*

All results were correct for the three parameters.

Sample 4: Pigfeed (blank)

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

PD for terrestrial invertebrates' particles:

- Labs 16 and 24 reported the presence of cuticle parts, muscles and other insect parts.

Two NRLs described the sample as containing mites.

Sample 5: Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder

All results were correct for the three parameters.

The presence of blood or haemoglobin powder was reported by only 10 NRLs (38 %).

4.1.1.3. Individual performances of NRLs in qualitative analysis

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

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

Terrestrial vert.			
lab code	**AC**	**SE**	**SP**
1, 2, 4, 5, 7, 8, 9, 12, 13, 14, 15,16, 17, 19, 20, 21, 22, 23, 25 and 26	1.000	1.000	1.000
3, 6, 10, 11, 18 and 24	0.800	1.000	0.667

Terrestrial inv.			
lab code	**AC**	**SE**	**SP**
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, 21, 22, 23, 25 and 26	1.000	1.000	1.000
16	0.800	1.000	0.750
24	0.600	1.000	0.500

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

Fish			
lab code	**AC**	**SE**	**SP**
all NRLs	1.000	1.000	1.000

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

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

Consolidated			
lab code	**AC**	**SE**	**SP**
1, 2, 4, 5, 7, 8, 9, 12, 13, 14, 15, 17, 19, 20, 21, 22, 23, 25 and 26	1.000	1.000	1.000
3, 6, 10, 11, 16 and 18	0.933	1.000	0.900
24	0.800	1.000	0.700

From the 26 NRLs, 19 performed excellently (73 %), 6 performed satisfyingly (23 %) and 1 was underperforming (4 %).

In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), the underperforming participant (lab 24) is asked to report on the origin of their errors as well as on the actions they will undertake to solve the problems.

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

Individual performances from the 3 participants outside the EU were assessed exactly as in the previous section (4.1.1.3). A ranking of those labs was prepared as well based on the consolidated accuracy. Results are to be found in Tables 8, 9 and 10.

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

Terrestrial vert.			
lab code	**AC**	**SE**	**SP**
30	1.000	1.000	1.000
28	0.800	1.000	0.667
27	0.600	0.500	0.667

Terrestrial inv.			
lab code	**AC**	**SE**	**SP**
30	1.000	1.000	1.000
28	0.800	1.000	0.750
27	0.600	0.000	0.750

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

Fish			
lab code	**AC**	**SE**	**SP**
30	1.000	1.000	1.000
27	0.800	1.000	0.667
28	0.800	0.500	1.000

Sample 1: Pigfeed + 0.1% feather meal + 1% egg

ND for terrestrial vertebrates:

- Lab 27 failed at detecting terrestrial animal remains.

PD for terrestrial invertebrates' particles:

- Lab 27 reported cuticle, mouth part, and legs

All results were correct for the fish parameter

Sample 2: Pigfeed (containing milk) + 1% salmon

PD for terrestrial vertebrates' particles:

- Lab 27 reported the presence of bones.
- Lab 28 reported having found muscles and hairs

In addition to these observations, Lab 30 declared this sample as positive for terrestrial vertebrates according to the finding of milk or milk products. Since the pigfeed was known to contain milk, the result was considered as correct.

All results were correct for the terrestrial invertebrates.

ND for fish particles:

- Lab 28 failed at detecting fish.

Samples 3: Poultry feed + 0.5% *T. molitor*

All results were correct for the terrestrial vertebrate parameter.

ND for terrestrial invertebrates:

- Lab 27 failed at detecting terrestrial invertebrates' remains.

PD for fish particles:

- Lab 27 reported fishbones and scales

Sample 4: Pigfeed (blank)

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

PD for terrestrial invertebrates' particles:

- Lab 28 reported the presence of invertebrates' parts without any description.

Few insect fragments, at levels < LOD were also reported by lab 30.

Sample 5: Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder

All results were correct for the three parameters.

Few insect fragments, at levels < LOD were also reported by lab 27 but without any description.

The presence of blood or haemoglobin powder was only reported by Lab 30. Nonetheless Lab 27 commented the sample as positive for blood plasma.

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

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

Consolidated			
lab code	**AC**	**SE**	**SP**
30	1.000	1.000	1.000
28	0.800	0.800	0.800
27	0.667	0.600	0.700

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

4.2. PCR results

4.2.1. Qualitative analyses from the NRLs

4.2.1.1. On the respect of the instructions

The NRLs seem to stick generally to the SOPs. Nevertheless, few labs do not use one of the EURL-AP recommended mastermixes : Lab 14 uses the TaqMan™ Universal PCR Master Mix (ThermoFisher Scientific) for the detection of ruminant DNA whereas it uses the Brilliant II QPCR Low ROX Master Mix (Agilent) for the detection of pig and poultry DNA. Lab 19 uses TaqMan™ Universal PCR Master Mix from Applied Biosystems™. Lab 25 encoded the Maxima Probe qPCR Master Mix (2x) from ThermoFisher Scientific. It has been also noticed that labs 6, 7, 20, 26 still use the Universal mastermix DMML-D2-D600 (Diagenode). Lab 18 used a mastermix provided by LaCar. Its reference number is identical to the one of the mastermix of Diagenode and, according to the lab, it would be the same.

4.2.1.2. Overview of results and global performance of the network

Table 12 summarizes the results provided by 26 NRLs for the five samples submitted to qualitative PCR analysis.

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

Sample	Material	n	AC		
			Ruminant	Pig	Poultry
1	Pigfeed + 0.1% feather meal + 1% egg	26	1.000	1.000	1.000
2	Pigfeed (containing milk) + 1% salmon	26	1.000	1.000	1.000
3	Poultry F + 0.5% *T. molitor*	26	n.a.	1.000	1.000
4	Pigfeed (blank)	26	1.000	1.000	1.000
5	Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder	26	1.000	1.000	0.962 (1)

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

On the overall results, only 1 deviation (0.3 % out of the 364 results) was recorded. With the ruminant and the porcine targets, the results are perfect. The rate of false results obtained with the poultry assay is 0.8 % which is fully acceptable.

Sample 1 : Pigfeed + 0.1% feather meal + 1% egg

The PCR results expected were the presence of poultry DNA only. All the results for the detection of ruminant, porcine and poultry DNA were correct.

Sample 2 : Pigfeed (containing milk) + 1% salmon

The PCR results expected was the presence of ruminant DNA and the absence pig and poultry DNA. No deviation was recorded.

Sample 3 : Poultry feed + 0.5% T. molitor

The PCR results expected were the absence of ruminant, pig and poultry DNA. All the results for the detection of porcine and poultry DNA were correct. 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.

Sample 4 : Pigfeed (blank)

The PCR results expected were the absence of ruminant, pig and poultry DNA. All the results recorded were correct.

Sample 5 : Fishfeed + 0.1% bovine PAP + 0.1% porcine haemoglobin powder

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

4.2.1.3. Individual performances of NRLs in qualitative analysis

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

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

Lab code	AC	SE	SP
1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 and 26	1.000	1.000	1.000
8	0.882	1.000	0.857

Excellent performances were recorded for 25 labs out of 26 NRLs (96 % of the NRLs) having no false result.

One lab (Lab 08) is satisfying. One positive deviation was recorded by Lab 08 for the detection of poultry DNA.

4.2.1.4. Cut-off quality control

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

Except lab 21, all the other participants reached the minimum criterion of 9.00 copies for the ruminant cut-off. The range of copies at the cut-off goes from 8.98 copies to 11.84 copies. The cut-off in cycles are comprised between 31.30 cycles and 37.60 cycles. The percentage of the labs with a cut-off corresponding to a number of copies > 10 for this proficiency test was 73.1 %.

For the pig cut-off, all the participants reached the minimum criterion set at 3.00 copies. The range of copies at the cut-off goes from 3.01 copies to 4.69 copies. The cut-off in cycles are comprised between 36.01 cycles and 42.12 cycles. Lab 19 reported a cut-off at 46.71 but this value seems to be an error. The percentage of the labs with a cut-off corresponding to a number of copies > 3.50 for this proficiency test was 61.5 %.

Considering the poultry cut-off, one NRL (Lab 26) did not reach the minimum criterion of 9.00 copies. Nevertheless, it did not impact its results as no deviation was recorded. The range of copies at the cut-off goes from 7.92 copies to 12.52 copies. The cut-off in cycles are comprised between 35.80 cycles and 43.20 cycles.

It must be noticed that some participants do not update their cut-offs since the PT 2023 or even before: Lab 01, 02, 04, 07 and 25 uses one or more than one cut-off since the PT 2022.

4.2.2. Qualitative analyses from the non-EU participants

4.2.2.1. Individual performances

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

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

Lab code	AC	SE	SP
28 and 30	1.000	1.000	1.000

Labs 28 and 30 obtained excellent results (no deviation).

4.2.2.2. Assessment of the cut-off values

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.

5. Discussion and conclusions

The overall performance for the microscopic detection of PAPs of the NRL network for the present study was optimal. The total error rate was of only 2 %. The number of excellent and satisfactory scores obtained within the network of NRLs reached respectively 73 % (54 % in 2023 [6] and 39 % in 2022 [7]) and 23 % (38 % in 2023 [6] and 42 % in 2022 [7]). The rate of underperforming NRLs for the present study was of 4 % (8 % in 2023 [6] and 19 % in 2022 [7]). Although this excellent level of performance, some points of attention still need a focus for global improvement of the proficiency.

The type of error depicted in this study is majorly positive deviations leading to specificity issues. The detailed analysis of the terrestrial vertebrates' parameter for sample 2 showed that all the 6 erroneous results are linked to the misidentification of salmon bones interpreted as terrestrial animal bones. This is a well-known and documented issue. Former proficiency tests and papers [8, 9, 10] have presented and discussed this confusion of salmon bones with terrestrial vertebrates' bones. This situation emphasizes the need to maintain the knowledge of histological features (bones in particular) to avoid pitfalls for operators. This continuous refreshment of knowledge is one of the dedicated responsibilities of feed microscopists.

Still concerning sample 2, about one third of the NRLs (7 on 26) reported the presence of dairy product, under the form of milk globules and/or lactose crystals. The choice of using a feed matrix containing milk product for this sample was to obtain a positive signal for ruminant by PCR analysis and not aiming at obtaining a positive result for terrestrial vertebrates based on this criterion. Therefore, during the homogeneity study bare attention was put on this presence, which even revealed to be not consistent during the observations (only a low number of lactose crystals were observed). Nevertheless, some NRLs enabled even to disclose this low presence.

Some issues related to erroneous insect findings remained limited. They involved only two participating NRLs. The detailed descriptions of the specific fragments (such as tracheal structures) largely excluded the possible presence of mites. However, a few mentions in the comments by four participants indicated uncertainty, leading them to either refrain from classifying the fragments as insects or not report them due to their rarity—placing them below any limit of decision.

Interestingly, this topic warrants further discussion towards more harmonized reporting. The current legal framework for official control focuses on the detection of prohibited materials in feed. More specifically, authorities aim to identify and disclose the presence of insect PAPs in ruminant feed. In this context, further detailed description in reporting may be necessary to distinguish the unauthorized use of insect PAPs from the incidental presence of other terrestrial invertebrates (e.g., weevils, mites). While these invertebrates are undesirable due to their impact on feed quality, their presence itself does not constitute an infringement of the feed ban.

Concerning results from the non-EU participants, encountered problems were comparable. The most frequent source of error was linked to specificity issues for terrestrial vertebrates for sample 2. Any other comparison attempt would be inappropriate since they are not bound to the same legal framework.

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 skills of the NRLs network are clearly confirmed. Only one deviation out of 364 results, representing 0.3 % of the results, is observed.

This means that 96 % of the NRLs perfectly managed the analyses of the set of samples while the remaining NRLs performed satisfactorily. No underperforming NRL was recorded despite the strict performance criterion (no deviation allowed) concerning the detection of ruminant DNA.

One result was kept out of assessment by the organisers due to divergent results obtained during the homogeneity study. Nevertheless, all the participants came to the same conclusion as the organisers: absence of ruminant DNA in the sample 3. Fundamentally, the removing of this parameter did not change the excellent performances of the network.

Acknowledgment

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

[8] Veys P, Berben G, Baeten V. 2011. EURL-AP interlaboratory study microscopy 2010. Final version. Gembloux (Belgium): European Union Reference Laboratory for Animal Proteins in feedingstuffs, Walloon Agricultural Research Centre.

[9] van Raamsdonk LWD, Pinckaers VGZ, Vliege JJM. 2012b. Animal proteins in feed. IAG ring test 2012. Report 2012.009. Wageningen: RIKILT; p. 39

[10] van Raamsdonk, L. W. D., Prins, T. W., van de Rhee, N., Vliege, J. J. M., & Pinckaers, V. G. Z. (2017). Microscopic recognition and identification of fish meal in compound feeds. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 34(8), 1364-1376. https://doi.org/10.1080/19440049.2017.1283711

Annex 1

Announcement letter

European Union Reference Laboratory for Animal Proteins in feedingstuffs

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Announcement of the EURL-AP proficiency test 2024/01 for the determination of Processed Animal Proteins (PAPs) in feed

Introduction

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

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

Objectives

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

The organizer team

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

Test material

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

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

General outline of the exercise

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

European Union Reference Laboratory for Animal Proteins in feedingstuffs

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Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : **6 September 2024**
- Sending of the sample boxes and communication of the instructions : **31 October 2024**

> ***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 : **29 November 2024**

Further information

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

or

- Dr Pascal VEYS
  EURL-AP Deputy Director
  ☎32 (0) 81 87 52 28
  🖷32 (0) 81 87 40 19
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Annex 2

Excel result report form

Proficiency Test Microscopy-PCR 2024/01

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
	Sample rank	1st	2nd	3th	4th	5th
	Sample N°					
		Light microscopy				
Light microscopy analyses	Terrestrial vertebrates particles					
	details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.*					
	Terrestrial invertebrates particles					
	details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.*					
	Fish particles					
	details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.*					
	Number of determinations					
	Free comment *Example : presence of unusual fragments....*					

PCR analyses

	PCR		
	Ruminant	Poultry	Pig
Cut-off at 15 *(5 for pig)* copies of the PCR platform used (in cycles)			
Copy number at the cut-off of the PCR platform used (in copies)			
Maxter mix used			
Sample N°			
Ruminant DNA			
Dilution 1 (e.g. 1 fold)			
Ct value replicate 1			
Ct value replicate 2			
Dilution 2 (e.g. 10 fold)			
Ct value replicate 1			
Ct value replicate 2			
Poultry DNA			
Dilution 1 (e.g. 1 fold)			
Ct value replicate 1			
Ct value replicate 2			
Dilution 2 (e.g. 10 fold)			
Ct value replicate 1			
Ct value replicate 2			
Pig DNA			
Dilution 1 (e.g. 1 fold)			
Ct value replicate 1			
Ct value replicate 2			
Dilution 2 (e.g. 10 fold)			
Ct value replicate 1			
Ct value replicate 2			
Free comment *Example : PCR inhibition....*			

Annex 3

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

Laboratory identification code :**1**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
1	291	Present	plasma, hydrolysed feather, bones	Absent		Absent		2
4	498	Absent		Absent		Absent		1
2	762	< LOD	bones	Absent		Present	muscle, fish bones	2
5	789	Present	bones	Absent		Present	muscle, fish bones, shell	2
3	801	Absent		Present	cuticule	Absent		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.	Number of determinations
1	471	Present	some bones, muscle fibers	Absent		Absent		1
5	501	Present	bones, muscle fibers, blood particles	Absent		Present	fish bones, cartilages, otolith, muscle fibers	1
4	570	Absent		Absent		Absent		1
2	600	Absent		Absent		Present	fish bones, muscle fibers	1
3	693	Absent		Present	cuticules	Absent		1

Laboratory identification code :**3**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	207	Absent		Present	cuticule, setae	Absent		1
1	453	Present	feather	Absent		Absent		1
2	654	Present	bones	Absent		Present	bones	1
4	678	Absent		Absent		Absent		1
5	771	Present	bones, muscle	Absent		Present	bones, muscle	1

Laboratory identification code :**4**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	45	Absent		Present	cuticular fragments setae	Absent		2
5	231	Present	bones cartilages muscles blood	Absent		Present	fishbones muscles cartilages	2
2	474	Present	milk powder derivate (whey)-lactose crystals	Absent		Present	fishbones otholites muscles	2
1	579	Present	bones milk powder muscles	Absent		Absent		2
4	768	Absent		Absent		Absent		2

regarding sample 579, we notice an unusual fragment, we found just a gill, sometimes the fishbones are quite similar to terrestial bones (poultry mainly), as can be seen on the website at micrograph collection so it is a contamination or some of the bones are fishbones so there would be fish also.

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.	Number of determinations
5	483	Present	bones, muscle fibers, cartilage	Absent		Present	scales, fishbones, cartilage, muscle fibers, otholithes, gills	1
1	507	Present	bones, cartilage	Absent		Absent		1
3	585	Absent		Present	muscle fibers, cuticles, setae	Absent		2
2	708	Absent		Absent		Present	scales, fishbones, cartilage, muscle fibers	1
4	930	Absent		Absent		Absent		1

Sample 483 : TMB + (result obtained on the flotate TCE/EP). The other samples are TMB-.

Laboratory identification code :**6**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	639	Absent		Present	cuticular fragments, muscles	Absent		
5	645	Present	bones, muscle fibers	Absent		Present	fish bones, muscle fibers	
1	705	Present	feathers,	Absent		Absent		
4	894	Absent		Absent		Absent		
2	942	Present	bones, muscle fibers	Absent		Present	fish bones, muscle fibers	

Laboratory identification code :**7**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
1	237	Present	Bones	Absent		Absent		2
5	321	Present	Bones	Absent		Present	Bones	1
3	423	Absent		Present	Particles from body	Absent		2
4	516	Absent		Absent		Absent		1
2	744	< LOD	Bones	Absent		Present	Bones	2

Laboratory identification code :**8**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
5	303	Present	Bones	Absent		Present	Fishbones, gills, scales, shells	2
2	528	Present	Milk	Absent		Present	Fishbones	2
3	729	Absent		Present	Cuticules, Tracheal system, muscles	Absent		2
1	741	Present	Bones	Absent		Absent		2
4	786	Absent		Absent		Absent		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.	Number of determinations
2	24	Absent		Absent		Present	fishbone, muscle	2
3	153	Absent		Present	chitin	Absent		1
1	435	Present	feather, plasma powder, bone	Absent		Absent		2
5	573	Present	haemoglobin powder, muscle, bone	Absent		Present	fishbone, muscle	2
4	840	Absent		Absent		Absent		1

Laboratory identification code :**10**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
5	123	Present	bones, blood particles	Absent		Present	bones, muscles, cartilage	1
2	294	Present	bones	Absent		Present	bones, muscles, scale	1
3	567	Absent		Present	insect particles (cuticule, muscles...)	Absent		1
4	750	Absent		Absent		Absent		1
1	849	Present	bones, muscles, feathers	Absent		Absent		1

Laboratory identification code :**11**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	531	Absent		Present	cuticules	Absent		1
2	582	Present	bones	Absent		Present	bones	2
1	651	Present	bones	Absent		Absent		1
5	717	Present	bones	Absent		Present	scales, bones	1
4	984	Absent		Absent		Absent		1

Laboratory identification code : **12**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
1	201	Present	bones, hydrolysed feathers, muscles	Absent		Absent		2
2	438	Absent		Absent		Present	fishbones, gills, muscles	2
3	513	Absent		Present	muscle, cuticule	Absent		2
5	519	Present	bones, muscles	Absent		Present	fishbones, muscles	2
4	552	Absent		Absent		Absent		1

Laboratory identification code : **13**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	171	Absent		Present	Insect cuticule	Absent		2
2	222	Present	Presence of milk in the form of lactose cristals	Absent		Present	Bones and a few gills fragments. Muscle Fibers	2
5	375	Present	Bones. Muscle fibers	Absent		Present	Bones (+ a few otolith). Muscle fibers	2
4	588	Absent		Absent		Absent		2
1	777	Present	Bones (not a lot but above LOD). Feathers	Absent		Absent		2

Laboratory identification code : **14**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
4	12	Absent		Absent		Absent		1
2	42	Present	lactose crystals	Absent		Present	fish bones, muscle fibres	2
3	117	Absent		Present	cuticle fragments, muscle fibres	Absent		1
5	195	Present	terrestrial bones, blood, muscle fibres	Absent		Present	fish bones, otolith, muscle fibres	2
1	795	Present	terrestrial bones, muscle fibres, feathers	Absent		Absent		2

Sample 195: TMB+H2O2 stain confirmed the presence of blood.
Sample 795:
Cystein stain confirmed the presence of feathers.
We noted the presence of clear globules, however TMB+H202 stain could not confirm their identity as plasma powder.
We noted the presence of 2 adult insect appendages in the final flotate fraction, over two determintaions; these were not considered an adulteration of the feed, but natural infestation.

Laboratory identification code : **15**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
5	177	Present	bones, muscle fibres, blood products it can't be exluded muscle fibres found only derive from terr. vertebrates and fish	Absent		Present	fishbones, scales, muscle fibres it can't be exluded muscle fibres found only derive from terr. vertebrates and fish	1
2	204	Present	milk products	Absent		Present	fishbones, scales, muscle fibres it can't be exluded muscle fibres found only derive from fish	1
1	219	Present	bones, muscle fibres, feathers it can't be exluded muscle fibres found only derive from terr. vertebrates	Absent		Absent		1
3	441	Absent		Present	cuticula, muscle fibres,… it can't be exluded muscle fibres found only derive from terr. invertebrates	Absent		1
4	660	Absent		Absent		Absent		1

samples 219 and 660 showed a mite investation

Laboratory identification code :**16**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
2	420	Absent		Absent		Present	muscles, fishbones, gills	1
3	711	Absent		Present	muscles, cuticule particles	Absent		1
5	861	Present	muscles, bones, blood	Absent		Present	muscles, fishbones, scales, otoliths	1
4	876	Absent		Present	muscles, heads, legs, eggs	Absent		1
1	921	Present	muscles, bones	Absent		Absent		1

Laboratory identification code :**17**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
2	258	Present	milk	Absent		Present	fish bones	2
1	381	Present	feathers	Absent		Absent		2
4	462	Absent		Absent		Absent		2
3	891	Absent		Present	fragments of cuticula, muscle fibres	Absent		2
5	969	Present	bones, hemoglobin	Absent		Present	fish bones	2

Laboratory identification code :**18**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
5	105	Present	bones, cartilage	Absent		Present	fishbones, cartilage, otolith, teeth, gills, muscles	1
4	120	Absent		Absent		Absent		1
1	165	Present	bones, cartilage and feather	Absent		Absent		1
2	348	Present	bones, cartilage	Absent		Present	fishbones, cartilage, muscles	2
3	747	Absent		Present	muscles and insect parts	Absent		1

In samples 120 and 165 there were mites in different stages of devlopmnent (egg, eggs with mites and part of the mites body). Since they were mites and in different developmental stages we have not categorized them as insect-PAP.

Laboratory identification code :**19**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	99	Absent		Present	insect cuticle	Absent		2
4	192	Absent		Absent		Absent		1
5	213	Present	bone, cartilage, muscle, blood	Absent		Present	fish bone, cartilage, muscle	2
1	363	Present	bone, feather, milk powder	Absent		Absent		2
2	834	Absent		Absent		Present	fish bone, muscle	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.	Number of determinations
2	78	Absent		Absent		Present	bones	1
4	696	Absent		Absent		Absent		1
3	783	Absent		Present	cuticule	Absent		1
1	867	Present	bones	Absent		Absent		1
5	951	Present	bones	Absent		Present	bones	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.	Number of determinations
3	81	Absent		Present	cuticula, legs	Absent		1
4	138	Absent		Absent		Absent		1
1	147	Present	bones, feathers	Absent		Absent		1
5	429	Present	bones	Absent		Present	bones, scales	1
2	852	Present	milk	Absent		Present	bones, scales	1

Laboratory identification code : **22**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	243	Absent		Present	cuticula, trachee,legs, muscle fibres	Absent		1
4	300	Absent		Absent		Absent		1
5	537	Present	bones, muscle fibres, cartilage	Absent		Present	fish bones , muscle fibres, cartilage, fish scales, gill, fish skin, otolith	2
2	564	Absent		Absent		Present	fish bones, cartilage, muscle fibres, fish skin	2
1	903	Present	feather meal, cartilage, muscle fibres	Absent		Absent		2

sample 300 and 903: mite particles

Laboratory identification code : **23**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
4	84	Absent		Absent		Absent		1
1	93	Present	Bones, muscle fibers	Absent		Absent		1
3	387	Absent		Present	Cuticle	Absent		1
2	690	< LOD	Bones	Absent		Present	bones, gills, scales, cartilage	2
5	879	Present	Bones	Absent		Present	Bones, gills, scales, cartilage, muscle fibers.	1

In the sediment of sample 93 we detected a low number of terrestrial bones (9 bones in one determination) in addition in the flotate of the same sample were detected muscle fibers and we have a suspicion for the presence of blood plasma. In the sediment of sample 690 were detected less than ten particles of terrestrial bones (7 particles) in two determinations (4 particles in the 1st and 3 particles in the 2nd determination). In the flotate were detected muscle fibers, as it is not possible to differentiate whether they originate from fish or terrestrial we didn't mention their presence in the table above. In sample 879 in addition to the above mentioned particles was detected blood in the flotate.

Laboratory identification code : **24**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
2	96	Present	bone, cartilage, muscle	Absent		Present	fishbone, cartilage, muscle	1
4	480	Absent		Present	'cuticle fragments, muscle	Absent		1
3	621	Absent		Present	cuticle fragments, tracheal structures, muscle	Absent		1
1	669	Present	bone	Present	'cuticle fragments, tracheal structures, muscle	Absent		1
5	843	Present	bone, blood, muscle, cartilage	Absent		Present	fishbone, gill, fish scale, blood, muscle, cartilage	1

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.	Number of determinations
5	15	Present	bones, muscle fiber	Absent		Present	bones	2
1	111	Present	bones, feathers	Absent		Absent		2
4	534	Absent		Absent		Absent		1
2	816	Absent		Absent		Present	bones	2
3	855	Absent		Present	cuticule	Absent		2

Laboratory identification code : **26**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
4	156	Absent		Absent		Absent		1
5	267	Present	bones,meat, blood Haemoglobin.	Absent		Present	bone, meat, scales, gills otoliths, cartilage	1
2	384	< LOD	bones	Absent		Present	bone, scales, cartilage, meat	2
1	615	Present	bone, feather, cartilage, plasma like particles	Absent		Absent		1
3	819	Absent		Present	mucels, cuticula. T.molitor	Absent		1

Laboratory identification code : **27**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
4	30	Absent		Absent		Absent		1
1	255	Absent		Present	ORAL CAVITY, CUTICULE, LEG	Absent		1
2	330	Present	BONES	Absent		Present	BONES	1
3	549	Absent		Absent		Present	BONES, SCALES	1
5	609	Present	BONES, FEATHER, MUSCLE FIBER	< LOD		Present	BONES, SCALES	1

Positive for the determination of blood plasma in the code sample 609

Laboratory identification code : **28**

Sample type	Sample N°	Terrestrial vert. part.	Details of terrestrial vert. part.	Terrestrial invert. part.	Details of terrestrial invert. part.	Fish part.	Details of fish part.	Number of determinations
3	63	Absent		Present		Absent		
5	159	Present	found muscle	Absent		Present		
1	561	Present		Absent		Absent		
4	606	Absent		Present		Absent		
2	780	Present	found muscle and hair	Absent		Absent		

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.	Number of determinations
5	33	Present	bones, blood (haemoglobin), muscles	Absent		Present	fishbones, otoliths, muscles	2
4	174	Absent		< LOD	insects - head, legs	Absent		2
3	333	Absent		Present	insect cuticule	Absent		2
1	345	Present	feathers	Absent		Absent		2
2	978	Present	milk: lactose crystals	Absent		Present	fishbones, muscles	2

Annex 4

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 :	35,43	43,20	40,48	cycles
Copy number at the cut-off :	10,16	9,05	3,52	copies
Master mix used :	Eurogenetec RT-QP2X-03			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
1	**291**	Absent	Present	Absent	
4	**498**	Absent	Absent	Absent	
2	**762**	Present	Absent	Absent	
5	**789**	Present	Absent	Present	
3	**801**	Absent	Absent	Absent	

Laboratory identification code : **2**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,35	38,16	38,97	cycles
Copy number at the cut-off :	10,82	9,10	3,45	copies
Master mix used :	Eurogentec qPCR Mastermix RT-QP2X-03			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
1	**471**	Absent	Present	Absent	
5	**501**	Present	Absent	Present	
4	**570**	Absent	Absent	Absent	
2	**600**	Present	Absent	Absent	
3	**693**	Absent	Absent	Absent	

Laboratory identification code : **3**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,49	35,76	37,14	cycles
Copy number at the cut-off :	11,41	11,65	3,79	copies
Master mix used :	Brilliant II QPCR Low ROX Master Mix, cat. N. 600806			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	**207**	Absent	Absent	Absent	
1	**453**	Absent	Present	Absent	
2	**654**	Present	Absent	Absent	
4	**678**	Absent	Absent	Absent	
5	**771**	Present	Absent	Present	

Laboratory identification code : **4**

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

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	**45**	Absent	Absent	Absent	
5	**231**	Present	Absent	Present	
2	**474**	Present	Absent	Absent	
1	**579**	Absent	Present	Absent	
4	**768**	Absent	Absent	Absent	

Laboratory identification code : **5**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	37,60	41,70	41,10	cycles
Copy number at the cut-off :	9,55	11,02	3,01	copies
Master mix used :	AGILENT - Ref : 600806			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
5	**483**	Present	Absent	Present	
1	**507**	Absent	Present	Absent	
3	**585**	Absent	Absent	Absent	
2	**708**	Present	Absent	Absent	
4	**930**	Absent	Absent	Absent	

Laboratory identification code : **6**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,07	38,44	39,08	cycles
Copy number at the cut-off :	9,28	9,43	3,06	copies
Master mix used :	DMMLD2D100 (GMO-UN-600, RT-QP2X-03			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	**639**	Absent	Absent	Absent	
5	**645**	Present	Absent	Present	
1	**705**	Absent	Present	Absent	
4	**894**	Absent	Absent	Absent	
2	**942**	Present	Absent	Absent	

Laboratory identification code : **7**

	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 (DMMLD2D600)			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
1	237	Absent	Present	Absent	
5	321	Present	Absent	Present	
3	423	Absent	Absent	Absent	
4	516	Absent	Absent	Absent	
2	744	Present	Absent	Absent	

Laboratory identification code : **8**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,21	38,41	38,06	cycles
Copy number at the cut-off :	10,90	9,10	3,50	copies
Master mix used :	Eurogentec- batch 215			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
5	303	Present	Present	Present	The concentration of poultry in this sample would appear to be very close to the cut-off, therefore the ct are variable between the different aliquots
2	528	Present	Absent	Absent	
3	729	Absent	Absent	Absent	
1	741	Absent	Present	Absent	
2	786	Absent	Absent	Absent	

Laboratory identification code : **9**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,04	36,89	37,81	cycles
Copy number at the cut-off :	11,76	11,54	4,03	copies
Master mix used :	Eurogentec RT-QP2X-03NR (Ruminant), Agilent Brilliant II low Rox mix (Poultry and Pig)			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
2	24	Present	Absent	Absent	
3	153	Absent	Absent	Absent	
1	435	Absent	Present	Absent	
5	573	Present	Absent	Present	
4	840	Absent	Absent	Absent	

Laboratory identification code : **10**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,83	38,12	40,22	cycles
Copy number at the cut-off :	11,93	11,37	3,83	copies
Master mix used :	Brilliant II QPCR Low ROX Master mix Lot 6742655			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
5	123	Present	Absent	Present	
2	294	Present	Absent	Absent	
3	567	Absent	Absent	Absent	
4	750	Absent	Absent	Absent	
1	849	Absent	Present	Absent	

Laboratory identification code : **11**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	31,30	36,07	36,01	cycles
Copy number at the cut-off :	12,77	11,24	3,48	copies
Master mix used :	Qpcr MasterMix EUROGENTEC			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	531	Absent	Absent	Absent	
2	582	Present	Absent	Absent	
1	651	Absent	Present	Absent	
5	717	Present	Absent	Present	
4	984	Absent	Absent	Absent	

Laboratory identification code : **12**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,73	38,15	38,73	cycles
Copy number at the cut-off :	10,93	9,05	3,23	copies
Master mix used :	Agilent Briljant IIQPCR Master Mix with Low Rox			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
1	201	Absent	Present	Absent	
2	438	Present	Absent	Absent	
3	513	Absent	Absent	Absent	
5	519	Present	Absent	Present	
4	552	Absent	Absent	Absent	

Laboratory identification code : **13**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	32,16	37,38	38,73	cycles
Copy number at the cut-off :	11,79	10,48	3,54	copies
Master mix used :	Eurogentec qPCR Mastermix			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	171	Absent	Absent	Absent	No PCR inhibition (test by adding 40cp/38.5cp/45cp plasmid to extract)
2	222	Present	Absent	Absent	No PCR inhibition (test by adding 40cp/38.5cp/45cp plasmid to extract)
5	375	Present	Absent	Present	No PCR inhibition (test by adding 40cp/38.5cp/45cp plasmid to extract)
4	588	Absent	Absent	Absent	No PCR inhibition (test by adding 40cp/38.5cp/45cp plasmid to extract)
1	777	Absent	Present	Absent	No PCR inhibition (test by adding 40cp/38.5cp/45cp plasmid to extract)

Laboratory identification code : **14**

	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); Poultry and Pig : Brilliant II QPCR Low ROX Master Mix (Agilent, product code 600806)			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
4	12	Absent	Absent	Absent	
2	42	Present	Absent	Absent	
3	117	Absent	Absent	Absent	
5	195	Present	Absent	Present	
1	795	Absent	Present	Absent	

Laboratory identification code : **15**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	37,19	39,70	41,06	cycles
Copy number at the cut-off :	10,03	10,64	3,62	copies
Master mix used :	Agilent Brilliant II QPCR Mastermix with Low ROX			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
5	177	Present	Absent	Present	
2	204	Present	Absent	Absent	
1	219	Absent	Present	Absent	
3	441	Absent	Absent	Absent	
4	660	Absent	Absent	Absent	

Laboratory identification code : **16**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	32,23	35,67	36,96	cycles
Copy number at the cut-off :	11,77	11,12	3,90	copies
Master mix used :	qPCR MasterMix Plus w/o UNG			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
2	420	Present	Absent	Absent	
3	711	Absent	Absent	Absent	
5	861	Present	Absent	Present	
4	876	Absent	Absent	Absent	
1	921	Absent	Present	Absent	

Laboratory identification code : **17**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,92	38,30	39,11	cycles
Copy number at the cut-off :	9,70	12,52	4,69	copies
Master mix used :	Brillant II qPCR Low Rox Master Mix			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
2	258	Present	Absent	Absent	
1	381	Absent	Present	Absent	
4	462	Absent	Absent	Absent	
3	891	Absent	Absent	Absent	
5	969	Present	Absent	Present	

Laboratory identification code : **18**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,18	37,10	38,13	cycles
Copy number at the cut-off :	9,14	9,50	3,02	copies
Master mix used :	LaCAR Universal mastermix			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
5	105	Present	Absent	Present	
4	120	Absent	Absent	Absent	
1	165	Absent	Present	Absent	
2	348	Present	Absent	Absent	
3	747	Absent	Absent	Absent	

Laboratory identification code : **19**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,85	42,66	46,71	cycles
Copy number at the cut-off :	9,67	9,74	3,15	copies
Master mix used :	AB\TaqMan Universal Master Mix			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	**99**	Absent	Absent	Absent	
4	**192**	Absent	Absent	Absent	
5	**213**	Present	Absent	Present	
1	**363**	Absent	Present	Present	
2	**834**	Present	Absent	Absent	

Laboratory identification code : **20**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	11,64	10,99	3,59	cycles
Copy number at the cut-off :	36,45	39,42	38,21	copies
Master mix used :	Diagenode Universal Mastermix			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
2	**78**	Present	Absent	Absent	
4	**696**	Absent	Absent	Absent	
3	**783**	Absent	Absent	Absent	
1	**867**	Absent	Present	Absent	
5	**951**	Present	Absent	Present	

Laboratory identification code : **21**

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

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	**81**	Absent	Absent	Absent	
4	**138**	Absent	Absent	Absent	
1	**147**	Absent	Present	Absent	
5	**429**	Present	Absent	Present	
2	**852**	Present	Absent	Absent	

Laboratory identification code : **22**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	34,15	35,80	36,60	cycles
Copy number at the cut-off :	11,15	11,37	3,75	copies
Master mix used :	Agilent Brilliant II low Rox Order no 600806-51			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	**243**	Absent	Absent	Absent	
4	**300**	Absent	Absent	Absent	
5	**537**	Present	Absent	Present	
2	**564**	Present	Absent	Absent	
1	**903**	Absent	Present	Absent	

Laboratory identification code : **23**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	35,76	36,35	36,06	cycles
Copy number at the cut-off :	10,05	9,22	3,51	copies
Master mix used :	Universal Master Mix DA216			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
4	**84**	Absent	Absent	Absent	
1	**93**	Absent	Present	Absent	
3	**387**	Absent	Absent	Absent	
2	**690**	Present	Absent	Absent	
5	**879**	Present	Absent	Present	

Laboratory identification code : **24**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,19	36,80	38,74	cycles
Copy number at the cut-off :	11,31	10,66	3,61	copies
Master mix used :	Brilliant II QPCR Low Rox Master Mix			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
2	**96**	Present	Absent	Absent	
4	**480**	Absent	Absent	Absent	
3	**621**	Absent	Absent	Absent	
1	**669**	Absent	Present	Absent	
5	**843**	Present	Absent	Present	

Laboratory identification code : **25**

	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	Poultry DNA	Pig DNA	Comment
5	**15**	Present	Absent	Present	
1	**111**	Absent	Present	Absent	
4	**534**	Absent	Absent	Absent	
2	**816**	Present	Absent	Absent	
3	**855**	Absent	Absent	Absent	

Laboratory identification code : **26**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	34,10	36,60	36,70	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	Poultry DNA	Pig DNA	Comment
4	**156**	Absent	Absent	Absent	
5	**267**	Present	Absent	Present	
2	**384**	Present	Absent	Absent	
1	**615**	Absent	Present	Absent	
3	**819**	Absent	Absent	Absent	

Laboratory identification code : **28**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	37,71	39,70	40,13	cycles
Copy number at the cut-off :	11,18	9,41	3,84	copies
Master mix used :	Brilliant II qPCR Low ROX master mix (Agilent)			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
3	**63**	Absent	Absent	Absent	
5	**159**	Present	Absent	Present	
1	**561**	Absent	Present	Absent	
4	**606**	Absent	Absent	Absent	
2	**780**	Present	Absent	Absent	

Laboratory identification code : **30**

	Ruminant	Poultry	Pig	
Cut-off at 15 (*5 for pig*) copies :	36,31	37,57	38,86	cycles
Copy number at the cut-off :	10,76	9,62	3,83	copies
Master mix used :	Brilliant II QPCR Low ROX Master Mix Agilent 600806			

Sample type	Sample N°	Ruminant DNA	Poultry DNA	Pig DNA	Comment
5	**33**	Present	Absent	Present	
4	**174**	Absent	Absent	Absent	
3	**333**	Absent	Absent	Absent	
1	**345**	Absent	Present	Absent	
2	**978**	Present	Absent	Absent	