EURL-AP PCR Implementation Test 2012

Final version

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Summary

The European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP) organised the present implementation test for assessing the ability of the NRL network with respect to the detection of ruminant proteins in feed using the ruminant PCR test developed by TNO Triskelion bv as validated by the EURL-AP. Total number of participants was 25 NRLs. One NRL declared just after reception of the samples to be unable to participate to the study due to the absence of PCR equipment in the lab. The study was based on a set of 10 blind samples. The sample set consisted of 4 feed samples (blanks or feed matrices fortified with terrestrial processed animal proteins) and 6 DNAs extracted from similar feed samples.

Four NRLs did not provide results in due time (at the deadline of 11 May 2012) and not even up to the end of May 2012. Results from the 21 remaining NRLs indicated an excellent performance for the PCR performed on the 6 extracts provided by the EURL-AP. These results confirmed what was obtained during the validation study providing once more evidence that the PCR step is fit for purpose. When preparation of the samples and DNA extraction steps have to be integrated in the analytical process, some laboratories with probably less experience had false positive results on the blank sample containing 1 % (w/w) of pig PAP. All samples containing ruminant PAP were however detected without problem which means that in a perspective of a partial lifting of the feed ban , the NRLs would be able with this method to detect ruminant PAP in feed if present at 0.1 % (w/w) in feedingstuffs. Corrective actions will be taken with the participants having had false positive results.

Keywords :

Processed animal proteins – Ruminant – PCR – Polymerase Chain Reaction - Implementation test – Qualitative analysis

1. Foreword

European Union Reference Laboratories (EURL) – formerly referred to as Community Reference Laboratories (CRL) – were created in order to ensure a high level of quality and a uniformity of the results provided by European control laboratories. On 29 April 2004, the European Parliament and the Council adopted the Regulation EC/882/2004 [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, the Commission Regulation EC/208/2011 [2], renewed the Walloon Agricultural Research Centre as European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP, http://crl.cra.wallonie.be). 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 organised this PCR interlaboratory study for the assessment of the implementation of a newly validated PCR method for the detection of ruminant proteins in feed.

2. Introduction

According to the TSE Roadmap II, alternative analytical methods to the classical microscopy able to detect and identify the species of processed animal proteins (PAPs) in animal feed are the main condition for a possible lifting of the extended feed ban [3]. The objective of the present implementation test is to evaluate performances of the network of 26 NRLs to detect the presence of ruminant processed animal proteins in feed using the newly validated PCR method [4].

3. Material and methods

3.1. Study organisation

Official announcement of the study was made on the 21st February 2012 through a PCR Roadmap letter sent to all participants.

Participants were the 26 NRLs of the EURL-AP network but one NRL withdrew its participation just after reception of the samples of the study due to a lack of equipment. A detailed list of the 26 participating labs is included in Annex 1.

On the 19th of March 2012, the ruminant PCR protocol was diffused as well as the calibrators allowing the determination of the cut-off value of a PCR platform and 5 samples that can be used as positive controls. The 3rd of April 2012, the remaining material (sets of 10 blind samples) for the implementation test was provided to the participants by express shipment. The 4th of April 2012, Excel report forms containing the instructions (Annex 2) and a questionnaire downloadable from the EURL-AP intranet were communicated to all participants.

Some general recommendations were delivered to the participants:

- 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.
- A summarized results sheet was automatically generated. Participants were asked to sign the summarized results sheet and to return it by fax and email to the EURL-AP. Only when both the Excel file and the fax were received by EURL-AP were results taken into consideration.
- The results had to be sent in both forms concomitantly to the EURL-AP by the 11th of May 2012 (the first deadline was the 2nd of May but on the request of the NRLs during the annual workshop in Berlin, an extension was accepted).

Twenty-one participants delivered their results in due on time. The four remaining participants did not provide any result before the deadline but not even by the 31st of May although they were asked by the EURL-AP to still communicate their results once they had it even after the deadline. As already mentioned above, one NRL resigned to participate just after reception of the samples because of a lack of equipment.

3.2. Material

3.2.1. Description of the samples

Different materials containing typical feed ingredients and/or processed animal proteins (PAPs) from various animal origins at different concentration levels ~ 0.1 % in weight have been prepared as shown in Table 1 (next page). Four feed samples were provided to extract their DNA according to the protocol imposed by the EURL-AP. The remaining six samples were DNAs ready to be used for the PCR out of which 5 had already been used in the validation study.

Each participating lab received about 10 g of the 4 feed samples and 100 µl of the 6 DNAs. A unique random number was assigned to each sample (Annex 3). Details of the samples are indicated in Table 1.

Table 1: Composition of the blind sample set used in the EURL-AP PCR Implementation Test 2012.

Sample	Material	Remark	
Feed samples			
1	0.1 % w/w sheep PAP in blank 1	1	
2	0.1 % w/w cattle PAP in blank 1	1	
3	0.1 % w/w cattle PAP in blank 1	1	(replicate of sample #2)
4	1 % w/w pig PAP in blank 1	1	
DNA extracts			
5	0.2 % w/w cattle PAP in blank 2	1	
6	0.1 % w/w cattle PAP in blank 2	1	
7	5 % w/w pig PAP in blank 3	1	
8	0.1 % w/w sheep PAP in blank 1	1	(DNA extracted from sample #1)
9	Blank 2 (100 % vegetal)	1	
10	0.1 % w/w cattle PAP in blank 2	1	(replicate of sample #6)
Total		**10**	

3.2.2. Materials used in the preparation of the samples

Different feed matrices were used for the preparation of the sample set:

- Blank 1: feed for sow (used in samples #1, #2, #3, #4 and #8);
- Blank 2: mix made of 60 % (w/w) of barley, 16 % (w/w) of maize, 16 % (w/w) of flax and 8 % (w/w) of alfalfa (used in samples #5, #6, #9 and #10);
- Blank 3: ground maize kernels (used in sample #7).

PAP used to spike the blank material were the following ones:

- Cattle PAP heat treated at 141 °C (used in samples #2, #3, #5, #6 and #10);
- Sheep PAP heat treated at 133 °C (used in samples #1 and #8);
- Pig PAP heat treated at 133 °C (used in samples #4 and #7).

3.2.3. Description about the DNA extracted samples

The samples #5, #6, #7, #9 and #10 correspond to DNA extracts already used in the validation study [4]. They consisted of material extracted by a CTAB method because of the large volumes of DNA to be provided to participants but were diluted in order to mimic Promega extracts and the comparisons made during the validation study showed they were not easier than extracts gained through the Promega extraction method advised to be used by the EURL-AP.

The DNA extract of sample #8 was obtained through the Promega DNA extraction protocol advised by the EURL-AP.

The samples preparation scheme is illustrated in Figure 1.

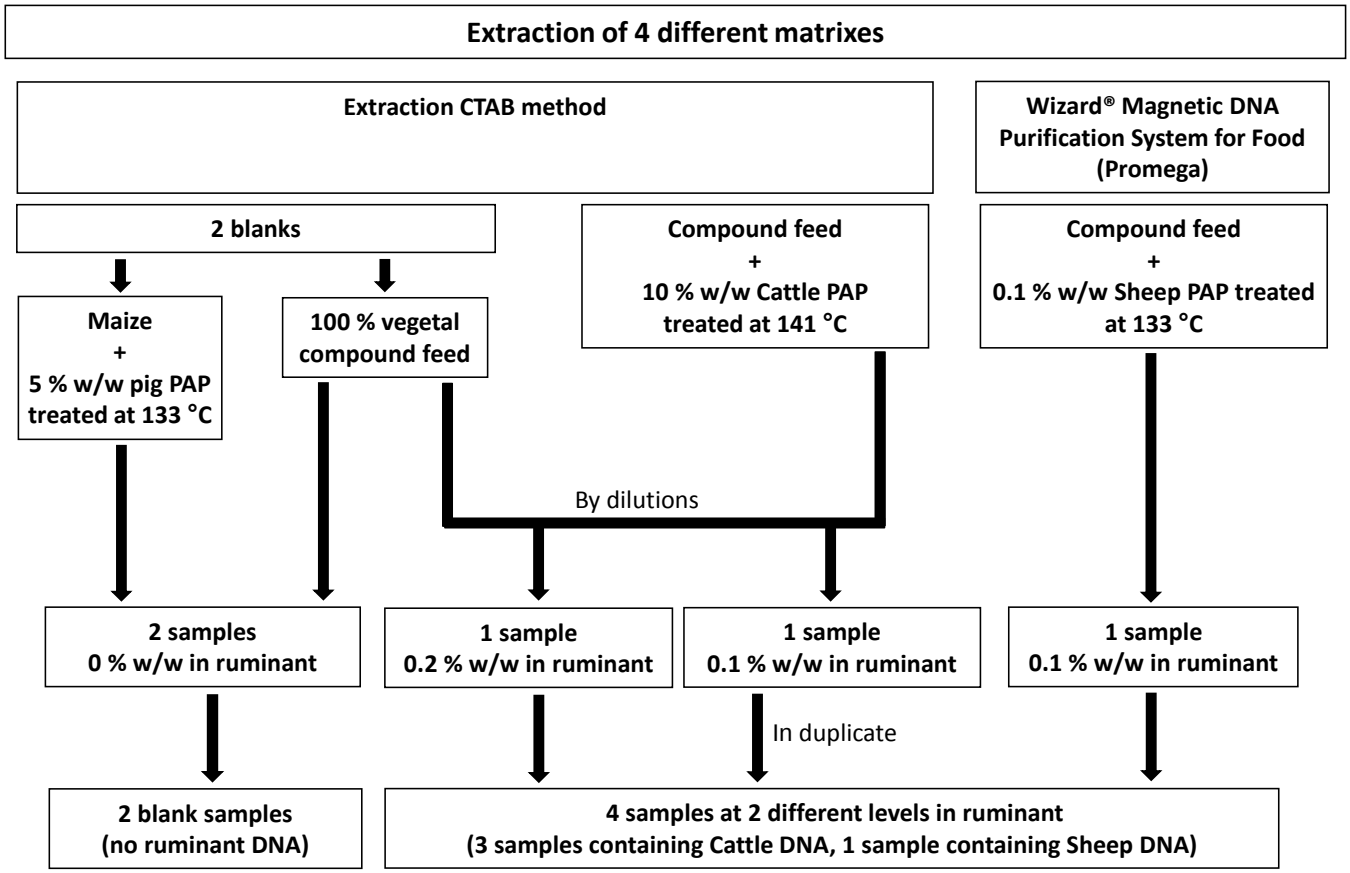


Figure 1: Production of the 6 blind DNA samples used in the EURL-AP PCR Implementation Test 2012.

3.3. Qualitative analysis

Qualitative analysis concerned the presence or absence of ruminant PAP material. These binary results were analysed by classical statistics: accuracy, sensitivity and specificity. All those statistics were expressed as fractions.

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

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

With :

PA : positive agreement (i.e. number of times detection was done when expected)
NA : negative agreement (i.e. number of times there was no detection when expected)
PD : positive deviation (i.e. number of times detection was done even though detection was not expected)
ND : negative deviation (i.e. number of times there was no detection even though detection was expected)

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

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

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

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

The *AC, SE* and *SP* were calculated separately for each laboratory for the estimation of its proficiency. A consolidated *AC* over both 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 method.

Results

Gross results from all participants are to be found in Annex 4.

3.4. Homogeneity study

For feed samples:
Ten replicates of each sample containing ruminant PAP were chosen randomly and were analysed using the ruminant PCR target. Per replicate, 2 DNA extracts were realised according the Promega protocol (http://intranet.crl.cra.wallonie.be/Document%20libraries/Protocols/EURL-AP%20DNA%20extraction%20protocols.pdf). In final, 20 Promega extracts were obtained per sample type to be analyzed (samples #2 and #3 are replicates of the same sample).
For the blank sample containing pig PAP, 2 aliquots were chosen randomly and analysed according to the same scheme as the other feed samples so that in final 4 Promega extracts were analysed by PCR with de pig target of the CRA-W. For this sample, 2 replicates were also analysed by light microscopy.
Moreover the pure pig PAP used in sample 4 has been tested with the ruminant PCR assay and all Ct values obtained (2 extractions, 16 PCR results) were always after the cut-off value (in other words the results were negative for the ruminant target).

For DNA extracts:
Sample #5 is a new dilution of a DNA extracted for the validation study according a CTAB protocol. It corresponds to samples #6 and #10 but two times less diluted for the content of cattle PAP.
Samples #6, #7, #9 and #10 were already used during the validation study of ruminant target and their homogeneity was checked for this study [4].
Sample #8 is a DNA extracted from sample #1 with the Promega protocol advised by the EURL-AP. The 20 DNAs analyzed to check the homogeneity of sample #1 were pooled to obtain sample #8.
Finally, each of the 6 DNA extracts was tested in blind four times.

Table 2: PCR results obtained with feed samples replicates

Sample	Material	Nr of test portion	Ruminant target	Bovine target	Sheep target	Pig target
1	0.1 % w/w sheep PAP in feed for sow	20	+	NT*	+	NT*
2 and 3	0.1 % w/w cattle PAP in feed for sow	20	+	+	NT*	NT*
4	1 % w/w pig PAP in feed for sow	4	-	NT*	NT*	+

*NT: not tested

Table 3: PCR results obtained with DNA samples replicates

Sample	Material	Nr of replicates	Ruminant
5	0.2 % w/w cattle PAP in blank 2	4	+
6 and 10	0.1 % w/w cattle PAP in blank 2	10* + 4	+
7	5 % w/w pig PAP in maize grains	10* + 4	-
8	0.1 % w/w sheep PAP in feed for sow	4	+
9	Blank 2 (100 % vegetal)	10* + 4	-

*tests done for the validation study of the ruminant PCR target

3.5. Qualitative analyses from the NRLs

3.5.1. Overview of results and performance of the method

Table 4 summarizes the results submitted by the 21 NRLs for the eight sample types submitted to qualitative analysis.

Table 4: Global results expressed as accuracy (AC) for the eight sample types

Sample	Material	Nr	AC
Feed samples			
1	0.1 % w/w sheep PAP	21	1.000
2 + 3	0.1 % w/w cattle PAP	42	1.000
4	1 % w/w pig PAP	21	0.762 (5)
DNA extracts			
5	0.2 % w/w cattle PAP	21	1.000
6 + 10	0.1 % w/w cattle PAP	42	1.000
7	1 % w/w pig PAP	21	1.000
8	0.1 % w/w sheep PAP	21	1.000
9	Blank 100 % vegetal	21	1.000

Accuracy means specificity in case of PD. In brackets the number of PD.
(Legend: Nr = number of observations).

The overall results, expressed in terms of global accuracy (AC), reveals a very good global performance. Nevertheless the number of false positive results in sample 4 (the only negative sample to be extracted was a blank containing 1 % in weight of pig PAP) is reaching 14 %.

3.5.2. Detailed review of results for each sample material

Incorrect results are detailed in this section.

They were all obtained with the feed sample containing 1 % w/w of pig PAP.

- **Lab 3:** not all replicates on sample 4 were positive but positive results dominated and the lab concluded that ruminant DNA was present.
- **Lab 11:** the negative extraction controls gave positive results (Ct<cut-off value) indicating a possible cross-contamination during the DNA extraction.
- **Lab 12:** the Ct values on sample #4 (38.62 and 37.95 cycles with the undiluted DNA) are very close to the cut-off value (38.74 cycles). The lab reported in its comments that the sample contained a very low concentration of ruminant PAP and that the dilutions of the DNA gave negative results.
- **Lab 21:** the lab did not use the recommended DNA extraction protocol but a CTAB protocol. Moreover the lab analysed test portions of 300 mg (instead of 100 mg) and recovered the DNA extracted in a final volume of 100 µl (instead of 300 µl). This explains the observed discrepancy.

- **Lab 25:** Apparently the problems do arise from the fact that the mastermix used gives positive signals.

3.5.3. Individual performances of NRLs in qualitative analysis

Individual performances were assessed for each participant by calculating the accuracy, sensitivity and specificity over the blind samples. This was performed separately for both the detection from feed samples and from DNAs. A ranking of the labs was prepared based on the consolidated accuracy.

Results are to be found in Tables 5 and 6.

Concerning the ability to detect ruminant material (Tables 5 and 6), 5 labs provided 1 incorrect result:

- PD for ruminant PAP in feed sample containing 1 % (w/w) pig PAP: labs 3, 11, 12, 21 and 25.

Tables 5 (left) and 6 (right): NRL proficiencies regarding the detection of ruminant material starting from feed samples and DNAs. Ranking follows AC values.

Feed samples			
Lab code	**AC**	**SE**	**SP**
1	1,000	1,000	1,000
2	1,000	1,000	1,000
4	1,000	1,000	1,000
5	1,000	1,000	1,000
6	1,000	1,000	1,000
7	1,000	1,000	1,000
8	1,000	1,000	1,000
9	1,000	1,000	1,000
13	1,000	1,000	1,000
14	1,000	1,000	1,000
16	1,000	1,000	1,000
19	1,000	1,000	1,000
20	1,000	1,000	1,000
22	1,000	1,000	1,000
23	1,000	1,000	1,000
26	1,000	1,000	1,000
3	0,750	1,000	0,000
11	0,750	1,000	0,000
12	0,750	1,000	0,000
21	0,750	1,000	0,000
25	0,750	1,000	0,000

DNAs			
Lab code	**AC**	**SE**	**SP**
1	1,000	1,000	1,000
2	1,000	1,000	1,000
3	1,000	1,000	1,000
4	1,000	1,000	1,000
5	1,000	1,000	1,000
6	1,000	1,000	1,000
7	1,000	1,000	1,000
8	1,000	1,000	1,000
9	1,000	1,000	1,000
11	1,000	1,000	1,000
12	1,000	1,000	1,000
13	1,000	1,000	1,000
14	1,000	1,000	1,000
16	1,000	1,000	1,000
19	1,000	1,000	1,000
20	1,000	1,000	1,000
21	1,000	1,000	1,000
22	1,000	1,000	1,000
23	1,000	1,000	1,000
25	1,000	1,000	1,000
26	1,000	1,000	1,000

A general ranking of the NRLs was performed on a consolidated evaluation including their proficiency in detecting ruminant material in both feed samples and DNAs (Table 7).

Table 7 illustrates the very good level of global performance (= consolidated AC superior or equal to 0.90, i.e. having no more than one false result) for 21 labs out of 26 NRLs or in other words for 81 % of the NRLs.

Table 7: General NRL proficiency regarding the detection of ruminant material. Ranking follows AC values as primary key and SE as second key.

Consolidated			
Lab code	**AC**	**SE**	**SP**
1	1,000	1,000	1,000
2	1,000	1,000	1,000
4	1,000	1,000	1,000
5	1,000	1,000	1,000
6	1,000	1,000	1,000
7	1,000	1,000	1,000
8	1,000	1,000	1,000
9	1,000	1,000	1,000
13	1,000	1,000	1,000
14	1,000	1,000	1,000
16	1,000	1,000	1,000
19	1,000	1,000	1,000
20	1,000	1,000	1,000
22	1,000	1,000	1,000
23	1,000	1,000	1,000
26	1,000	1,000	1,000
3	0,900	1,000	0,667
11	0,900	1,000	0,667
12	0,900	1,000	0,667
21	0,900	1,000	0,667
25	0,900	1,000	0,667

4. Conclusions

This study is the first assessment of the proficiency level in PCR methods of the NRL network. However out of the 26 participants, only 21 finally participated by submitting results. Looking globally at these results sent to the EURL-AP, 76.2 % of the participating NRLs (16 labs out of 21) obtained a very good global performance and had no false result. 23.8 % of the participating NRLs (5 labs out of 21) had one false positive result.

Different conclusions can be made:

1. The PCR step appears to be well implemented in the NRLs and no false result was recorded with the DNAs provided by the EURL-AP. This gives further evidence after the validation study that the PCR step as such is certainly fit for purpose. The link can be done with the validation study as most of the samples used were identical.
2. The DNA extraction and/or the preparation steps before the PCR have to be improved in 5 NRLs that are most probably less experienced with these steps. From additional information obtained after the deadline, the false positive results are mainly due to problems of cross-contamination. This problem could sometimes have been detected by the lab after the analysis of their negative extraction controls. Moreover, one of the labs did not use the appropriate DNA extraction method.
   Nevertheless, the importance of these false positive results remains difficult to estimate as the sample concerned was the only one to be negative for the presence of ruminant DNA among the samples to be extracted by the labs. Corrective actions will be undertaken with those labs having obtained false positive results (sending of a new sample set).
3. All the positive samples (with a ruminant content of 0.1 % in w/w) to be used in DNA extraction in this study could be detected successfully by all the NRLs having submitted results. This means that in a perspective of a partial lifting of the feed ban, the NRLs would be able with this method to detect ruminant PAP in feed if present at 0.1 % (w/w) in feedingstuffs.

Five NRLs (19.2 % of the whole NRL network) were not able to send any results. One NRL (Lab 24) will not be able to participate to another proficiency test in 2012 due to absence of appropriate equipment to perform the analyses. Three NRLs (Lab 10, 15 and 17) argued financial problems and delays to obtain the appropriate reagents or extraction kits. For the last NRL (Lab 18), the delay is due to software problems.

Acknowledgments

We are grateful to the EURL-AP staff and the participants for their fruitful collaboration.

References

[1] EU. 2004. Commission Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Official Journal of the European Union L 165, 30/04/2004: 1-141.

[2] EU. 2006. 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] The TSE Roadmap 2 - A Strategy paper on Transmissible Spongiform Encephalopathies for 2010-20. Communication from the Commission to the European parliament and the Council. Brussels, 16/07/2010, COM(2010)384 final. http://www.fsai.ie/uploadedFiles/Legislation/FSAI_-_Legislation/2010/07_jul2010/EU_Communication_TSE.pdf

[4] Validation study of a real-time PCR method developed by TNO Triskelion bv for the detection of ruminant DNA in feedingstuffs. Final report. June 2012. Olivier Fumière, Aline Marien, Gilbert Berben.

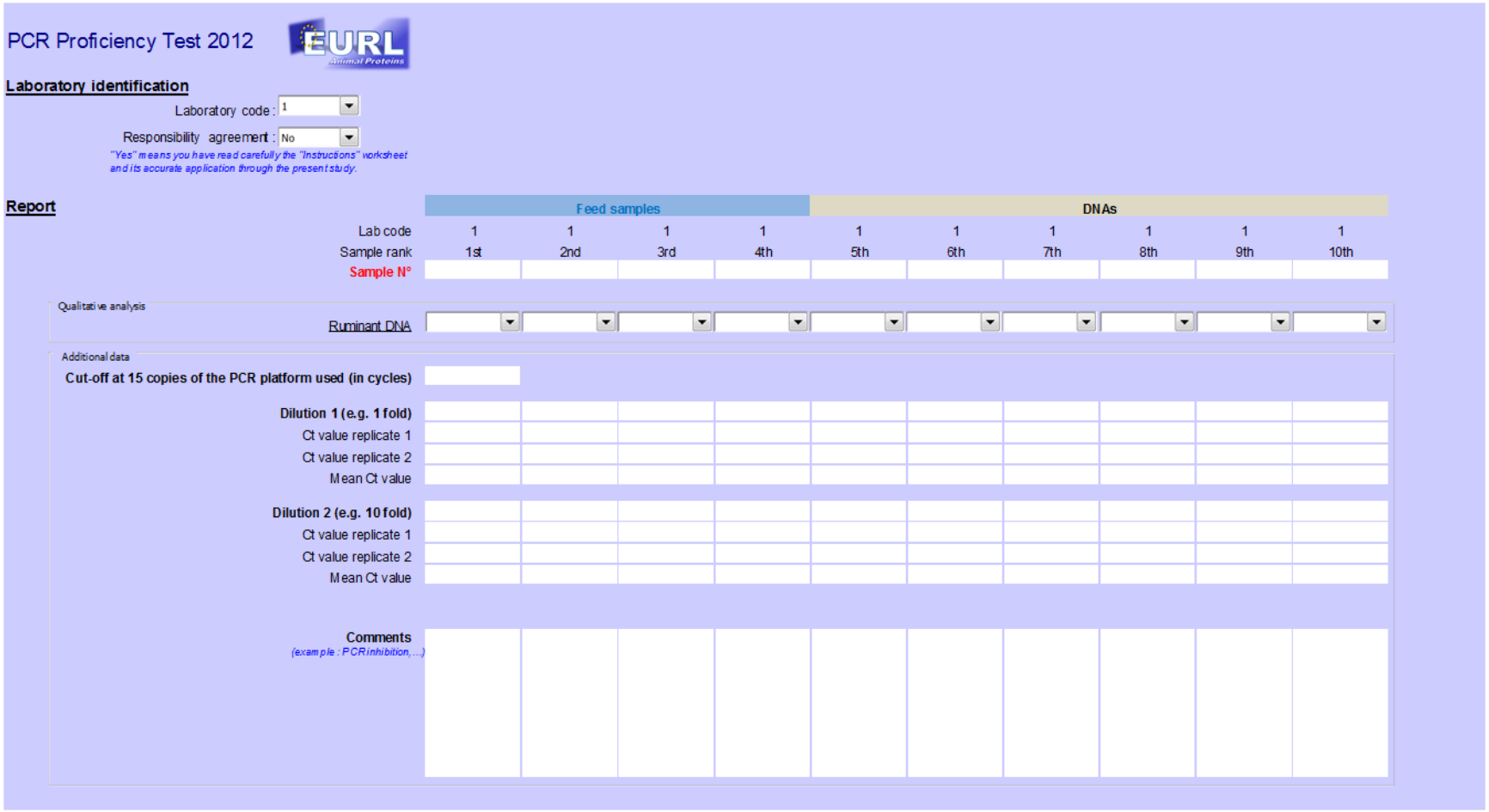
Annex 1

List of participants

Country	Institute Name
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
Cyprus	Cyprus Veterinary Services
Czech Republic	Central Institute of sampling and testing in Agriculture
Denmark	Danish Veterinary and Food Administration
Estonia	Veterinary and Food Laboratory
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
Hungary	Central Agricultural Office-Directorate Food and Feed Safety-Central Feed Investigation Lab.
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 Veterinary Laboratory
Luxemburg	Agroscope Liebefeld-Posieux Research Station (Switzerland)
Netherlands	RIKILT Institute of Food Safety, Wageningen UR
Poland	National Veterinary Research Institute
Portugal	Laboratorio Nacional de Investigaçao Veterinaria
Romania	Hygiene Institute of Veterinary Health
Slovakia	State Veterinary and Food Institute
Slovenia	Veterinary Faculty-National Veterinary Institute-Unit for pathology of animal nutrition and environmental hygiene
Spain	Laboratorio Arbitral Agroalimentario
Sweden	National Veterinary Institute, Department of Animal Feed
United Kingdom	Animal Health and Veterinary Laboratories Agency

Annex 2

Excel result report form



PCR Proficiency Test 2012

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

	Feed samples				DNAs					
Lab code	1	1	1	1	1	1	1	1	1	1
Sample rank	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
Sample N°										
Qualitative analysis										
Ruminant DNA										
Additional data										
Cut-off at 15 copies of the PCR platform used (in cycles)										
Dilution 1 (e.g. 1 fold)										
Ct value replicate 1										
Ct value replicate 2										
Mean Ct value										
Dilution 2 (e.g. 10 fold)										
Ct value replicate 1										
Ct value replicate 2										
Mean Ct value										
Comments *(example : PCR inhibition,...)*										

Annex 3

Composition of sample sets

	Lab number																								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
Blind samples																										
Feed samples																										
0.1 % w/w cattle PAP	1	8	11	14	20	21	25	32	35	38	44	45	49	56	59	62	68	69	73	80	83	86	92	93	97	104
0.1 % w/w cattle PAP	2	5	12	16	17	23	26	29	36	40	41	47	50	53	60	64	65	71	74	77	84	88	89	95	98	101
0.1 % w/w sheep PAP	3	6	9	13	19	22	27	30	33	37	43	46	51	54	57	61	67	70	75	78	81	85	91	94	99	102
1 % w/w pig PAP	4	7	10	15	18	24	28	31	34	39	42	48	52	55	58	63	66	72	76	79	82	87	90	96	100	103
DNA extracts																										
0.2 % w/w cattle PAP	200	199	198	342	341	340	194	193	346	345	190	189	188	349	348	347	192	191	344	336	197	196	195	339	338	337
0.1 % w/w cattle PAP	201	210	214	218	222	226	233	236	245	249	253	257	261	269	271	280	284	288	292	331	304	306	315	319	323	327
1 % w/w pig PAP	202	206	215	219	223	229	234	237	241	250	254	258	264	268	272	276	285	289	293	334	303	307	311	320	324	328
0.1 % w/w sheep PAP	203	207	211	220	224	227	231	238	242	246	255	259	262	266	273	277	281	290	294	332	301	308	312	316	325	329
Blank 100 % vegetal	204	208	212	216	225	230	232	239	243	247	251	260	265	267	274	278	282	286	295	335	302	309	313	317	321	330
0.1 % w/w cattle PAP	205	209	213	217	221	228	235	240	244	248	252	256	263	270	275	279	283	287	291	333	305	310	314	318	322	326

Annex 4

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

Laboratory identification code :	**1**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	39,2

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	1	Present	2	31	No Ct	31	10	30	32	31	PCR inhibition
2	2	Present	2	33	No Ct	33	10	31	31	31	PCR inhibition
3	3	Present	2	31	41	36	10	29	29	29	PCR inhibition
4	4	Absent	2	40	44	42	10	39	39	39	PCR inhibition
5	200	Present	2	32	32	32	10	34	34	34	
6	201	Present	2	33	33	33	10	35	35	35	
7	202	Absent	2	No Ct	41	41	10	No Ct	44	44	
8	203	Present	2	29	30	29	10	30	31	30	
9	204	Absent	2	No Ct	41	41	10	41	40	40	
10	205	Present	2	33	33	33	10	35	36	35	

Laboratory identification code :	**2**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	37,438

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	5	Present		35	34	34	10	36	37	36	Dilution 1: 25ng/µL DNA. Diltion 2: 2,5 ng/µL DNA.
2	6	Present		31	29	30	10	32	32	32	Dilution 1: 25ng/µL DNA. Diltion 2: 2,5 ng/µL DNA.
3	7	Absent		41	41	41	10	40	41	41	Dilution 1: 25ng/µL DNA. Diltion 2: 2,5 ng/µL DNA.
4	8	Present		33	34	34	10	36	38	37	Dilution 1: 25ng/µL DNA. Diltion 2: 2,5 ng/µL DNA.
5	199	Present		34	33	34	10	37	37	37	
6	206	Absent		42	41	41	10	40	39	41	
7	207	Present		34	34	34	10	33	33	33	
8	208	Absent		40	41	40	10	39	39	39	
9	209	Present		34	34	34	10	38	37	38	
10	210	Present		34	34	34	10	37	37	37	

Laboratory identification code :	**3**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	37,286

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	9	Present	1	26	26	26	10	29	28	29	
2	10	Present	1	34	34	34	10	36	36	36	sample a priori not homogenous, 4 DNA extractions and 10 PCR performed : detection of ruminant DNA 6 times on 10.
3	11	Present	1	32	32	32	10	34	34	34	
4	12	Present	1	31	31	31	10	33	33	33	
5	198	Present	1	30	30	30	10	34	34	34	
6	211	Present	1	32	32	32	10	30	30	30	PCR inhibition
7	212	Absent	1	40	41	40	10	39	41	40	
8	213	Present	1	31	32	31	10	35	35	35	
9	214	Present	1	31	31	31	10	34	35	35	
10	215	Absent	1	44	39	41	10	45		45	

Laboratory identification code :	**4**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	37,537508

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	13	Present	1	31	31	31	10	32	32	32	
2	14	Present	1	35	35	35	10	37	36	37	
3	15	Absent	1	43	43	43	10	40	40	40	
4	16	Present	1	35	35	35	10	35	36	36	
5	216	Absent	1	40	41	40	10	41	40	40	
6	217	Present	1	35	35	35	10	38	37	38	
7	218	Present	1	35	34	34	10	38	38	38	
8	219	Absent	1	41	42	42	10	41	40	41	
9	220	Present	1	34	34	34	10	33	33	33	
10	342	Present	1	33	34	34	10	37	37	37	

Laboratory identification code :	**5**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	36,235397

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	17	Present	1	32	32	32	10	35	35	35	
2	18	Absent	1	39	38	38	10	39	39	39	
3	19	Present	1	27	27	27	10	30	30	30	
4	20	Present	1	31	31	31	10	34	34	34	
5	221	Present	1	32	32	32	10	35	35	35	
6	222	Present	1	32	32	32	10	35	35	35	
7	223	Absent	1	40	42	41	10	40			Sample 223 - Ct value replicate 2 (10x) is undetermined - no signal in 50 cycles
8	224	Present	1	32	32	32	10	30	30	30	
9	225	Absent	1	40	38	39	10	41	41	41	
10	341	Present	1	30	31	31	10	34	33	34	

Laboratory identification code :	**6**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	37,07353

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	21	Present	1	31	31	31	10	33	33	33	
2	22	Present	1	27	27	27	10	29	29	29	
3	23	Present	1	30	30	30	10	32	32	32	
4	24	Absent	1	40	40	40	10	42	45	43	
5	226	Present	1	33	33	33	10	36	36	36	
6	227	Present	1	32	32	32	10	32	32	32	PCR inhibition in 1 fold dilution (ct values!)
7	228	Present	1	32	32	32	10	36	36	36	
8	229	Absent	1	45			10				flat signal
9	230	Absent	1	41	41	41	10	45	45	45	flat signals in 10 fold dilution
10	340	Present	1	31	31	31	10	35	35	35	

Laboratory identification code :	**7**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	35,95

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	25	Present	10	31	31	31	50	33	33	33	
2	26	Present	10	32	31	31	50	34	33	33	
3	27	Present	10	27	27	27	50	28	28	28	
4	28	Absent	10	40	40	40	50		44	44	
5	194	Present	2	30	30	30	8	32	32	32	
6	231	Present	2	29	29	29	8	29	29	29	PCR inhibition
7	232	Absent	2	39	40	39	8				
8	233	Present	2	31	31	31	8	33	33	33	
9	234	Absent	2	40	40	40	8				
10	235	Present	2	31	31	31	8	32	33	33	

Laboratory identification code :	**8**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	38,2

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	29	Present	1	32	32	32	10	36	36	36	
2	30	Present	1	30	28	29	10	33	31	32	
3	31	Absent	1	36	37	36	10	40	46	43	
4	32	Present	1	31	31	31	10	35	35	35	
5	193	Present	1	28	27	28	10	29	30	30	
6	236	Present	1	28	27	27	10	28	30	29	
7	237	Absent	1	42	37	39	10	40	42	41	
8	238	Present	1	27	27	27	10	24	25	25	
9	239	Absent	1	39	42	40	10	38	39	42	
10	240	Present	1	28	27	28	10	32	32	32	

Laboratory identification code :	**9**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	36,506298

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	33	Present		33	32	33	10	35	34	35	
2	34	Absent		43	47	45	10	39	39	39	
3	35	Present		36	34	35	10	37	34	36	
4	36	Present		36	35	36	10	37	37	37	
5	241	Absent		44	45	44	10	44	46	45	
6	242	Present		35	35	35	10	35	36	35	
7	243	Absent		41	44	42	10	42	40	41	
8	244	Present		35	35	35	10	39	39	39	
9	245	Present		36	36	36	10	39	39	39	
10	346	Present		34	35	35	10	38	38	38	

Laboratory identification code :	**11**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	37,796146

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	41	Present	1	33	33	33	10	35	36	35	
2	42	Present	1	33	32	32	10	34	34	34	
3	43	Present	1	30	30	30	10	32	32	32	
4	44	Present	1	32	32	32	10	34	34	34	
5	190	Present	1	34	34	34	10	37	36	37	
6	251	Absent	1	42	41	41	10	42	40	41	
7	252	Present	1	35	35	35	10	39	40	39	
8	253	Present	1	35	35	35	10	38	37	37	
9	254	Absent	1	43	41	42	10	41	39	40	
10	255	Present	1	34	33	34	10	33	33	33	

Laboratory identification code :	**12**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	38,74

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	45	Present	1	29	30	30	10	32	33	32	
2	46	Present	1	26	27	27	10	29	31	30	
3	47	Present	1	31	35	33	10	33	32	32	
4	48	Present	1	39	38	38	10	40	40	40	Very low concentration of ruminant PAP in the sample, dilutions gave negative results
5	189	Present	1	30	32	31	10	34	33	33	
6	256	Present	1	31	31	31	10	34	34	34	
7	257	Present	1	31	31	31	10	34	34	34	
8	258	Absent	1	39	40	39	10	44	50	47	Undiluted samples gave only two positive results of six replicates; in repetition three positive of 12 replicates (together c. 28 % positive). This was interpreted as negative results because the PAP in the sample seems to be << LOD.
9	259	Present	1	32	32	32	10	29	29	29	Certain amount of pcr-inhibition because the ruminant target was copied more effectly in the diluted sample.
10	260	Absent	1	40	39	39	10	50	41	45	Undiluted samples gave only one positive result of six replicates; in repetition one positive of 12 replicates (together c. 11 % positive). This was interpreted as negative results because the PAP in the sample seems to be << LOD.

Laboratory identification code :	**13**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	34,66

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	49	Present	1	31	29	30	10	34	32	33	Regarding the cut-off determination used for the computation we obtained efficiencies between approximately
2	50	Present	1	30	31	30	10	33	33	33	80% and 90%. We also observed relative high variations of the Ct values within the three calibrators. This might be
3	51	Present	1	28	26	27	10	30	29	30	due to the fact that these calibrators are plasmids, knowing that they can stick to the surface of the tube, hence influence
4	52	Absent	1	37	37	37	10	38	37	37	the concentration. The discrepancies of our obtained delta Ct values to the theoretical
5	188	Present	1	33	32	32	10	36	36	36	value (3.32) based on the information we have about this method are difficult to explain and certainly demand furhter
6	261	Present	1	33	33	33	10	36	36	36	investigations. We analyzed all ten samples independently two times and we obtained no significant differences.
7	262	Present	1	29	29	29	10	29	29	29	
8	263	Present	1	33	33	33	10	36	36	36	
9	264	Absent	1	37	37	37	10	39	39	39	
10	265	Absent	1	37	37	37	10	38	38	38	

Laboratory identification code :	**14**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	36,21

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	53	Present	1	31	31	31	10	33	32	33	
2	54	Present	1	28	27	27	10	29	28	29	
3	55	Absent	1	41	38	39	10	40	39	39	
4	56	Present	1	32	32	32	10	33	33	33	
5	266	Present	1	33	32	33	10	31	31	31	PCR Inhibition
6	267	Absent	1	45	42	43	10	40	39	40	
7	268	Absent	1	39	41	40	10	42	38	40	
8	269	Present	1	31	31	31	10	36	37	37	
9	270	Present	1	32	32	32	10	36	36	36	
10	349	Present	1	30	30	30	10	35	36	35	

Laboratory identification code :	**16**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	38,02

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	61	Present	1	32	31	32	10	35	34	34	
2	62	Present	1	37	35	36	10	38	37	37	PCR inhibition
3	63	Absent	1	40	42	41	10	41	42	41	
4	64	Present	1	36	35	36	10	38	36	37	PCR inhibition
5	276	Absent	1	40	39	39	10	40	40	40	
6	277	Present	1	37	37	37	10	37	36	36	PCR inhibition
7	278	Absent	1	39	40	40	10	38	39	39	
8	279	Present	1	36	37	36	10	39	39	39	
9	280	Present	1	36	37	37	10	39	39	39	
10	347	Present	1	35	35	35	10	38	38	38	

Laboratory identification code :	**19**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	37,29

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	73	Present		33	34	34	10	37	38	37	
2	74	Present		34	35	35	10	37	38	37	
3	75	Present		33	34	33	10	35	36	36	
4	76	Absent		42	42	42	10	43	43	43	
5	291	Present		32	33	33	10	38	37	37	
6	292	Present		33	33	33	10	37	38	38	
7	293	Absent		39	40	40	10				
8	294	Present		32	33	32	10	35	35	35	
9	295	Absent		41	43	42	10				
10	344	Present		32	31	31	10	35	34	35	

Laboratory identification code :	**20**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	36,71

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	77	Present	10	32	32	32	40	34	34	34	
2	78	Present	10	29	29	29	40	31	30	31	
3	79	Absent	10	38	38	38	40	40	44	42	
4	80	Present	10	32	32	32	40	35	34	35	
5	331	Present	10	36	35	35	40	37	38	38	
6	332	Present	10	30	30	30	40	31	31	31	
7	333	Present	10	36	35	35	40	37	38	38	
8	334	Absent	10	39	39	39	40	39	39	39	
9	335	Absent	10	41	42	42	40	39	38	39	
10	336	Present	10	34	34	34	40	37	36	36	

Laboratory identification code :	**21**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	37,8

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	81	Present	1	26	26	26	10	26	26	26	
2	82	Present	1	36	37	37	10	35	36	36	Weak positive
3	83	Present	1	28	28	28	10	28	28	28	
4	84	Present	1	28	29	29	10	29	29	29	
5	197	Present	1	30	30	30	10	33	33	33	
6	301	Present	1	30	30	30	10	29	29	29	
7	302	Absent	1	38	38	38	10	44	45	45	
8	303	Absent	1	40	40	40	10	41	43	42	
9	304	Present	1	31	31	31	10	34	34	34	
10	305	Present	1	31	31	31	10	34	34	34	

Laboratory identification code :	**22**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	38,31

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	85	Present	1	27	29	28	10	28	30	29	
2	86	Present	1	31	33	32	10	33	35	34	
3	87	Absent	1	40	39	39	10	42	41	42	
4	88	Present	1	32	32	32	10	34	33	34	
5	196	Present	1	32	32	32	10	36	35	36	
6	306	Present	1	34	34	34	10	38	37	37	
7	307	Absent	1	41	41	41	10		45	23	
8	308	Present	1	32	32	32	10	30	30	30	PCR inhibition
9	309	Absent	1	41	40	40	10	44	45	45	
10	310	Present	1	34	34	34	10	38	37	37	

Laboratory identification code :	**25**
Responsibility agreement :	**Yes**
Cut-off at 15 copies :	35,7

	Sample N°	Ruminant DNA	Dilution 1	Ct value 1	Ct value 2	Mean Ct value	Dilution 2	Ct value 1	Ct value 2	Mean Ct value	Comment
1	97	Present	1	32	32	33	10	34	34	34	
2	98	Present	1	33	33	33	10	34	34	34	
3	99	Present	1	27	28	27	10	30	30	30	
4	100	Present	1	33	33	33	10	35	35	35	
5	321	Absent	1	39	38	38	10				
6	322	Present	1	36	35	35	10	36	37	37	
7	323	Present	1	35	35	35	10	36	36	36	
8	324	Absent	1	36		36	10	37		37	
9	325	Present	1	34	34	34	10	32	32	32	inhibition
10	338	Present	1	34	34	34	10	37	35	36	