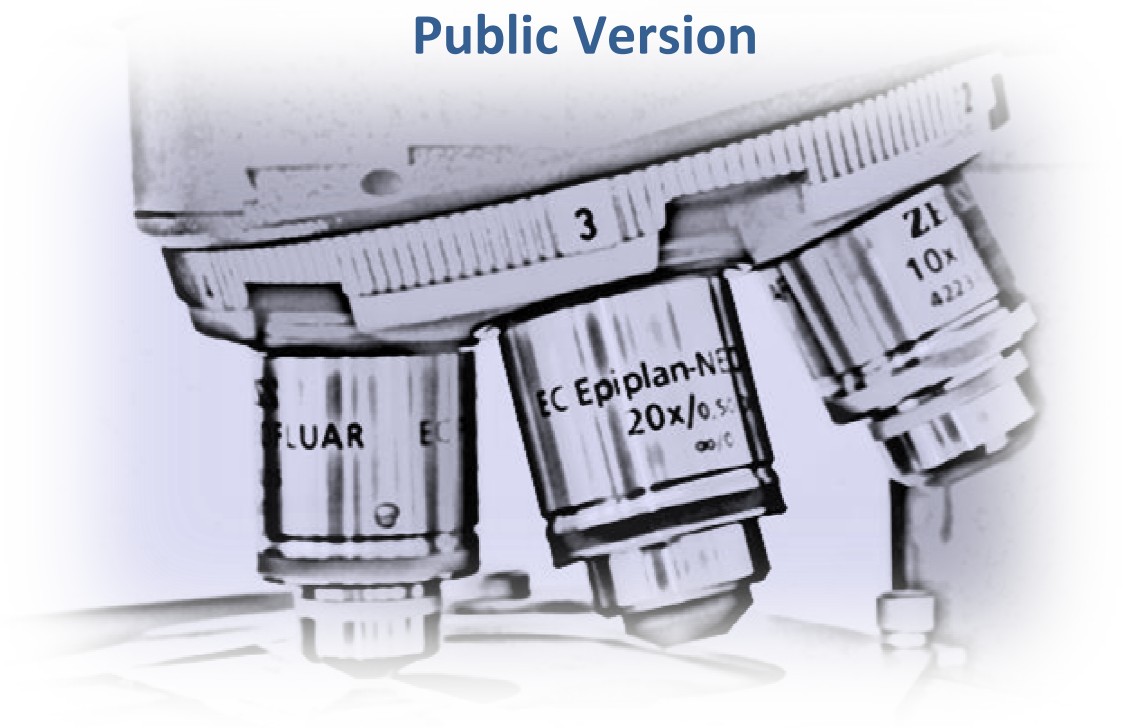
# The European Union Reference Laboratory for the detection of animal proteins in feedingstuffs

**Walloon Agricultural Research Centre – CRA-W (Belgium)**

# 2011 Annual report

## Public Version



# 1 Scientific advice and support to the European Commission (34 p/m)

## 1.1 Provide scientific and technical assistance to the European Commission in relation to the development of EC feed legislation. (2 p/m)

- ***Meeting of January 2010: meeting in Gembloux with DG-Sanco: The validation of the revised method from Annex VI of EC/152/2009 for the standardization of the method (not the principles) was performed through the EURL-AP interlaboratory study. The study took place on autumn 2010 but results were analysed during the first half year 2011. In agreement with a modification of the feed ban to a species-to-species ban, the new protocol can be integrated with use of an alternative method such as PCR. Proposals of operational schemes combining the use of light microscopy and PCR were presented and commented during the annual workshop.***

  ***The EURL-AP hosted the EC representative, Mr Martial Plantady, for a day (27$^{th}$ of May 2010) in order to show him the technical details of the implementation of the microscopy and PCR methods.***

- ***As it was asked in January to think about a text to add to the annex describing the PCR method in the new regulation lifting partially the ban on use of PAP in feed, the EURL-AP had to think on how to solve this while on the other hand the PCR should remain in some way an open technique, i.e. without mandatory assays. The only possible way to achieve this is to provide guidelines in the form of acceptance and performance criteria that a method should reach.***

  ***Examples do exist for instance in GMO detection but in a less broad context, this should be enlarged here to that topic. The assessment by the EURL-AP of the ruminant PCR method of TNO Triskelion provides an opportunity to define all parameters that should be considered.***

## 1.2 Upon the request of the European Commission or in order to fulfil his role as

EURL, participate in international fora/committees relating to the detection of animal proteins in feedstuffs (EFSA, WHO/FAO, JRC, etc.) with eventual presentations to prepare for it. As up to 2 European or international missions/year are foreseen in support to DG Sanco and/or EURL-AP activities. (1 p/m)

- ***Working Group TSE meeting (Brussels, 13th of April 2011): presentation to the Member States by DG-Sanco of a first draft of the new regulation in which partial lifting of the ban on use of PAP in feed is considered. EURL-AP was asked to highlight how analytical aspects have to be considered in that context. The main conclusion is that not all cases will be considered at the start of this new regulation. EURL-AP will try to cover approximately 95% of possible situations.***

- ***Scientists from the EURL-AP were invited by two French committees to present the state of the art of the analytical tools for the detection of the PAPs in the framework of a possible lifting of the feed ban. Dr Gilbert Berben gave a lecture to the working group of the French National Food Council (CNA, Paris, 18th of May) and Dr Olivier Fumière did the same exercise for a meeting of a working group of the French Agency for Food, Environmental and the Occupational Health & Safety (ANSES, 5th of July). These two working groups had to give their advice to the French authority on the possible re-introduction of the animal proteins in feed.***

- ***Dr Vincent Baeten participated to the annual meeting of FEFAC held in Bruges (8th of June 2011). During this meeting, a presentation on the status of the development of the microscopic and PCR methods has been given.***

- ***SCoFCAH meeting (Brussels, 26th of September 2011): presentation to the Member States by EURL-AP of the current situation and the remaining work schedule as regards validation/development of analytical techniques able to determine species origin of PAPs and of discussion on a draft proposal***

***amending annex VI of regulation (EC) N°152/2009 laying down the methods of sampling and analysis for the official control of feed.***

- ***Dr Gilbert Berben attended the "Technical meeting EURLs food safety" organized by DG-Sanco in Brussels during the afternoon of 15 December 2011. As the program integrated a part on 'molecular analyses' and because bacteria might in some circumstances also be interesting to analyse in order for instance to determine the geographical origin of a product (e.g. a fish meal), it was thought that participating to this meeting for which the EURL-AP received an invitation might be helpful (e.g. for instance with respect to setting up a database as also indicated in the program of the meeting). In fact, it was especially intended for those EURLs working on pathogenic bacteria or items related to that. Besides the fact that it helps to know who is working on what, it had little interest for the EURL-AP.***

- ***The EURL-AP team (Dr Pascal Veys and Dr Olivier Fumière) were involved in the organisation and participation of the BTSF (Better training for safer food) platform on TSEs as coordinated by JVL Consulting. The EURL-AP was requested to prepare lectures (1) on the light microscopic detection of PAP according current legislation, (2) on the PCR method implementation and (3) on the alternative methods for future improvement of the existing methods. In 2011, Dr Pascal Veys and Dr Olivier Fumière presented these lectures two times in Gembloux, Belgium (June and November), and two times in Manchester, UK (September and December). The total number of participant – majorly official control veterinarians from EU and foreign countries – for these sessions was reaching 120 people. The involvement of the EURL-AP in these BTSF workshops will be continued in 2012.***

1.3 Upon the request of the European Commission or in order to fulfil his role as EURL, participate in meetings for the standardisation of analytical methods relating to the detection of animal proteins in feedstuffs and their implementation (CEMA, ISO/CEN, OIE, IAG, etc.). Up to 3 European missions/year are foreseen in support to DG Sanco and/or EURL-AP activities. (2 p/m)

- ***The EURL-AP was present at the IAG annual meeting, held in Krefeld, Germany during the 7, 8 & 9 of June 2011. A lecture about the results of the EURL-AP validation study on a revised protocol for Annex VI of EC/152/2009 and future implementation scheme with PCR analysis was done.***

- ***From 30th April to 5th May 2011 the EURL-AP participated in the AOCS 102nd Annual meeting, which was held in Cincinnati, USA. Under the title "Defining and characterizing limits of detection for qualitative results: A realistic challenge? Study case in feed microscopy for PAPs detection", the lecture presented an original approach developed and tested for determining limits of detection in such situations. Methodology and model was applied to the reference microscopic method for the detection of processed animal proteins in the framework of TSE prevention in the EU. Results were examined within the issue on future application of the present total feed ban.***

- ***On 27 and 28 of September the EURL-AP attended the IAG autumn meeting in Hamburg Germany. Dr Pascal Veys presented a lecture on the future combination of methods (light microscopy – PCR) for the detection of animal proteins in feed.***

1.4 To actively participate in technical and scientific support of the European Commission in the context of incidents or crises linked to incorrect use of animal proteins. (3 p/m)

1.5 To keep at EURL-AP the highest standard possible of technical skill, scientific

awareness and quality management under accreditation (ISO17025) on analytical methods for detection, quantification and identification of animal proteins in feed ingredients and in feedingstuffs. To maintain and extend the accreditation scope of the EURL-AP lab. (14 p/m)

*1.5.1 To maintain scientific awareness in general about techniques that might be helpful in relation to topics of interest of the EURL-AP*

*1.5.2 To maintain the accreditation scope*

*1.5.3 Maintenance of the competences of the EURL-AP team and formation of the new recruits*

*1.5.4 Extend the accreditation scope of the EURL-AP by including additional animal targets DNA in the scope for PCR analyses*

*1.5.5 Submission of the dossier for the accreditation of the organisation of interlaboratory studies*

- ***In June 2011 the EURL-AP was submitted to an audit by the Belgian accreditation body, BELAC. No non-conformity was notified by the auditor. In September 2011 a new in depth revised protocol for the light microscopic detection of PAPs was inserted in the accreditation. This new protocol goes beyond the requirements of Annex VI of EC/152/2009 for an even more reliable detection. A protocol for the correct utilisation of the microscope was also written and entered into the accreditation scope in October 2011.***

- ***EURL-AP subscribed to a proficiency test (PT) scheme for the detection of animal proteins in animal feed by PCR organised by the VLA, Luddington, UK: 4 sets of 5 samples to be analysed per year. The sets of samples distributed in January, July and October were successfully analysed. The set of April was cancelled by the organizers.***

- ***EURL-AP subscribed also to proficiency tests for the detection of animal proteins in animal feed by classical microscopy organised by the VLA. Sets of 4 samples were received in March, June, September and November 2011. The results obtained by classical microscopy were successful.***

- ***The Ring Test IAG for the implementation of the microscopic method was also done; results are not available so far.***

  ***Samples of all proficiency tests are always analysed in parallel by classical microscopy and PCR like any other sample that would be analysed by the EURL-AP. In 2011, results of both methods were always consistent.***

- ***New recruits, Stephane Brichard, Marie Collard and Alison Pier joined the EURL-AP team. Ms Collard is replacing Isabelle Fissiaux who left the institute; she will be enforcing the microscopy lab and partly the PCR team. Mr Brichard is a new laboratory operator reinforcing the microscopy lab. They both started working at the microscopy laboratory on the 1st of August 2011. At this date Ms Pier was joining the PCR team as a technician. Intense training was provided to them.***

### 1.6 On the request of DG SANCO or the NRLs, to perform analyses on samples with disputed results. (12 p/m)

- ***In 2011, several request of official counter analysis were submitted by NRLs:***
  - ***1 fishmeal sample, from NRL1 was analysed on February 2011***
  - ***2 samples of ovine rendered material, from NRL2 were analysed on February 2011***
  - ***2 fishmeal samples from NRL3 were analysed on April 2011***
  - ***1 fishmeal sample from NRL4 was analysed on May 2011***
  - ***1 fishmeal sample from NRL5 was analysed on June 2011***
  - ***1 blood meal sample from NRL6 was analysed on October 2011***
  - ***1 compound feed from NRL7 was analysed on December 2011***

- ***Some analyses are also performed for helping NRLs in their decision making. Those analyses were not subjected to official reports. In 2011 the questions for information request and help from the NRLs have mainly dealt with fish feed and fish meals (e.g. salmon). Exchanges of micrographs were frequently***

***used as well as the consultation of the EURL-AP micrograph collection.***

1.7 Organisation in collaboration with TAIEX of a training/workshop for the candidate and potential countries (Turkey, Croatia, Iceland, FYR Macedonia, Albania, Bosnia and Herzegovina, Montenegro, Serbia). (0 p/m)

- ***In December 2011 Dr Pascal Veys prepared a lecture for a TAIEX for Bosnia which will take place in Sarajevo in January 2012.***

## 2 Coordination of activities of NRL network (11 p/m)

2.1 Maintenance and update of EURL website (internet/intranet) to disseminate and share information with NRLs and others stake holders. (5 p/m)

*2.1.1 Information collection and validation*

*2.1.2 Maintenance of the website*

*2.1.3 Update and test of the information system and validation*

- ***During the 12 months period, 5811 web pages from the public site were visited through 2275 visits by 810 unique visitors coming from 67 countries and 321 network locations throughout the world.***
  ***Regarding the intranet, 10228 hits were recorded during this period. 8 new NRL members joined the network and no member left.***
  ***For the public website, all those statistics are at the same level against the same period in 2010. However, the hits number registered on the intranet is 3200 hits lower than in 2010. That can be explained by the little updates regarding the EURL-AP micrograph collection which can be considered as established.***

- ***Monthly updates were carried out. Maintenance tasks were provided to maintain security and confidentiality systems and to operate backup.***

- ***In January 2011 the new public website was launched. Its content is intended to deliver a more detailed information on animal proteins detection in feed***

***and the role played by the EURL-AP. In this way it focused on the 5 axes of activities. The ICT team choose for a new managing platform allowing an easiness of content management: since its launching several new pages have been added. The public website will also provide updated information to the Member States' organisations wanting to have a clear vision on the methods used as well as the combinations of methods which will be required for the future policy on the feed ban according to TSE Roadmap II. In the second half year, on request of DG Sanco for better information of the Member States, new pages have been added with the respective advantages and drawbacks of each analytical method. In addition information on the future implementation of a possible combination of different detection methods were added. The new website includes also for downloadable formats of all scientific reports realised by the EURL-AP (proficiency tests, interlaboratory studies, evaluation of methods). The efforts spent for realising this new website will enforce the visibility of the EURL-AP and its network of partners.***

## 2.2 Prepare and send a six-months newsletter for NRLs. (1 p/m)

***Two newsletters were prepared and diffused among the NRLs in 2011: Newsletter 11 on May 2011. The 11th newsletter's content consists in the minutes of the 5th EURL-AP annual workshop which was held in Vienna the 6th and 7th of April 2011. This newsletter represents a workshop summary, including comments, decisions and brief abstract of the lectures.***

***Newsletter 12 on December 2011. The newsletter's content announced the 6th Annual Workshop which will be held in Berlin in 2012. It also informed the network on the ongoing proficiency tests (microscopy and GTH preparation) and also explained the planned 2012 program for the PCR development of methods and implementation of those methods by the NRLs. Several other announcements for conferences and meeting were included as well as information on the BTSF activities.***

***Newsletters 11 and 12 are attached to this report.***

## 2.3 Organisation of the annual EURL-AP meeting/workshop (3 p/m)

### *2.3.1 Organisation of the 5th annual EURL-AP workshop*

### *2.3.2 Preparation of the agenda*

### *2.3.3 Invitation of the attendees*

### *2.3.4 Realisation of the workshop*

### *2.3.5 Minutes of the annual workshop*

- ***The 5th EURL-AP annual workshop was held on 6th and 7th of April 2011 in Vienna, Austria. Once again, as in 2010, the EURL-AP team didn´t have to deal with the logistic organisation (meeting room renting, catering, hotel, etc.) as this time it was taken in charge by the Austrian NRL.***
    - ***After being meticulously prepared, the programme of the first day of meeting included the following items of presentation and discussions: AGES presentation and Austrian NRL network organisation and activities, a summary of the 2010 activity report of the EURL-AP 2010, the results of the 2010 EURL-AP interlaboratory study (on the microscopic method), the revision of the protocol: implementation of LOD data and minor improvements, presentation of the organization of a collaborative study on GTH detection which should take place in Autumn 2011, presentation about the EURL-AP imaging services, trainings on microscopy, and new supporting tools and the new EURL-AP website. Identification of blood products in feed by light microscopy was discussed. The day ended with the presentation of the TSE Road map II, the expectations of the network and a brief discussion about it.***
    - ***The second day of the workshop was mainly focused on PCR and integration of microscopy and PCR. The lectures included the following items: PCR enquiry of 2010-status of PCR in the network, a presentation done by the German NRL about the DNA extraction from meat and bone meal that was identified by them as a bottleneck in PCR analysis, PCR-training: presentation and first return, Practical issues of the start-up of PCR in the Belgian NRL. The morning session continued with the EURL-AP results of the PCR 2011 interlaboratory study, the status of the***

***development and the validation of PCR tests at the EURL-AP, combined approach: microdissection and PCR: first results from 2 labs, as well as the integration of microscopy and PCR in case of partial lifting of the feed ban on PAPs. The day and the workshop finished with the activity program for 2011 and the workshop conclusions.***

***This year representatives of both, the microscopy method and the PCR method were invited to the workshop. In most cases, 2 persons from each Member State were present, in other cases, the representative of the two methods turned out to be the same person. No representatives from a Member State1 were present; request for clarifying this absence was sent to the NRL1. Their explanation is still awaited.***

***Minutes of the workshop were recorded in the 11th EURL-AP newsletter.***

## 2.4 Supply information, scientific advices and protocols to NRLs, testing laboratories, detection, quantification and identification of animal proteins in feed ingredients and feedingstuffs. (0 p/m)

This task has been merged with task 3.3

- ***The EURL-AP is often contacted by NRLs in order to get protocols, or asked for advice on interpretation of pictures which are sent to the EURL-AP via e-mail. Such scientific advice becomes really a routine activity for the EURL-AP team. It is a testimony of the good communication between the NRL network and the EURL-AP.***

- ***In order to share as much as possible our research results obtained by the EURL-AP with the NRL network, all the PowerPoint presentations from the 5th annual workshop and from the trainings sessions were made available on the Intranet in pdf format.***

- ***EURL-AP continued its PCR training program launched in December 2010. Five NRLs (Bulgaria, Czech Republic, France, Hungary and Ireland) were invited and participated to a second session taking place from the 15th to the 17th of***

*February 2011. Two other training sessions were organised in September (13-15 September) and in October (4-6 October). This time, the participants were invited on a voluntary basis because at the Vienna Workshop some NRLs expressed the wish to attend the same training as the one that was considered mandatory by the EURL-AP for some NRLs identified for their lack of experience in PCR with the responses they provided to a survey of the summer 2010. Four NRLs participated to each of these two sessions: Austria, Spain, Poland and Lithuania in September; Slovenia, Finland, Latvia and Estonia in October. After five training sessions, a total of 19 NRLs already came in the EURL-AP facilities for a PCR training. The remaining NRLs (Denmark, Germany, Italy, Luxemburg, Sweden, The Netherlands and United Kingdom) feel confident enough with PCR and therefore had no need for training on this issue by the EURL-AP. They can indeed be considered as experienced PCR labs.*

*For the PCR too, the EURL-AP is more and more often contacted by NRLs but also by private companies in order to get information on protocols, or asked for advice (e.g. cost and characteristics of equipments, organization of the laboratory ...).*

*The protocol for the detection of bovine DNA in feed describing extensively the primers and probe sequences as well as the thermal program was distributed to the participants of the training sessions.*

- *Since the 19th of April, the same protocol is also available to the NRLs on the intranet of the EURL-AP website.*

- *Based on specific demands of some NRL's, other PCR methods designed by the EURL-AP (fish, pig) were transmitted to those NRLs having asked for it (they were not transmitted to all as they are not considered as completely ready for the network).*

- *In addition to the trainings and the protocols distributed, a DVD was finalized*

***during the summer. It describes the PCR analysis through video sequences of all analytical steps. The EURL-AP looked for a native English speaker to record the audio comments. Mr James Choiseul from the Irish NRL kindly accepted this job. One copy of the DVD was distributed to all the NRLs during the training sessions of September and October as well as sent by postal mail. This initiative was very appreciated by some NRLs (Bulgaria, Cyprus, Hungary, Ireland, Italy, Romania and Slovakia).***

- ***On the request of the Belgian NRL, a demonstration with a magnetic particles processor for the DNA extraction, the MagMAX Express 96 from Applied Biosystems, was organized in the EURL-AP facilities.***

2.5 Participate in the annual EURL Directors co-ordination meeting. (0 p/m)

- ***Instead of the normal annual EURL Director coordination meeting, in 2012 the EURLs were invited to a meeting held in Brussels on 16$^{th}$ of December where the results of the assessment of the several EURLs has been explained as well as the new administrative and financial rules. Vincent Baeten and Gilbert Berben attended this meeting. Moreover, as a follow up to this meeting, actions have been taken to address the items where the EURL-AP was considered as less performing : a SOP for follow up of underperforming NRLs was written (it will be applicable as of 1st January 2012) and a feed-back form for the EURL-AP training sessions was established (yet applicable for the PCR trainings which were hold in 2011).***

2.6 Prepare the six months and annual reports of activities according to the report guidelines transmitted by DG SANCO. (2 p/m)

***Redaction of the first 6-months report (January 2011- June 2011) done.***

***Redaction of the annual report (January 2011 – December 2011) done.***

## 3 Interlaboratory studies and quality assurance. (38 p/m)

3.1 Coordinate the preparation, reception, storage, maintenance and distribution to national reference laboratories (NRL) of samples containing animal proteins derived from different species and in particular from fish, poultry, pigs and ruminants to be used as reference materials or to carry out comparative testing. This task includes the preparation of the samples for the interlaboratory studies. (12 p/m)

*3.1.1 Definition of the needs*

*3.1.2 Collection of the raw materials*

*3.1.3 Control of the raw materials*

*3.1.4 Production of the samples*

*3.1.5 Test of the homogeneity of the samples produced*

*3.1.6 Report on the produced samples*

*3.1.7 Distribution of the samples*

- ***The FASFC (Belgian Federal Agency for the Safety of the Food Chain) has provided the CRA-W with 54 samples collected by official inspectors at different industrial plants, among them: samples of pig meal, milk replacer, vitamin E, blood meal and more.***

  ***All those samples have to be handled accordingly, stored in the correct place, analysed by PCR and encoded in the internal database. During the second half year the microscopic and PCR analyses were performed by the EURL-AP team.***

- ***CCL-Nutricontrol (Veghel, The Netherlands) provided on request of the EURL-AP several new samples of what has to be considered as pure poultry PAP coming from different rendering plants in the EU. This is important with respect to verification of performances of PCR methods dedicated to detection of poultry material.***

3.2 Organize interlaboratory study for the determination of PAPs in feed using classical microscopy. (9 p/m)

*3.2.1 Redaction of the report of the EURL-AP interlaboratory study of autumn 2010.*

*3.2.2 Definition (with the collaboration of the DG-Sanco) of the objectives of the ring trial to perform at the end of 2011.*

*3.2.3 Preparation of the interlaboratory study 2011.*

*3.2.4 Invitation of the NRLs to participate.*

*3.2.5 Packaging and sending of the samples. (cf. task 3.1)*

*3.2.6 Collection of the results.*

- ***Data from the EURL-AP ILS 2010 were analysed and compiled in a report in January 2011. A first working document version of the report was posted for download on the Intranet by 11th March 2011. The document as well as the results were discussed together with the NRLs at the 5th EURL-AP annual workshop in Austria. A final version of the report was prepared and communicated to the NRLs end of April. For reminder, the 2010 interlaboratory study for the NRL (not for the non-EU) was focusing mainly on the validation of the revised microscopic method from annex VI of EC/152/2009 as discussed from last 4th EURL-AP workshop held in Turin for the standardization of the method. The proposed modifications of the protocol had to be applied: "negative (LOD)" rule; options on stereomicroscope, sieving, 3g-10g sample, flotate; 6 slides before declaring negative. The study report clearly confirmed the validation of the new protocol and the general improvement of the results as obtained by the NRLs compared to previous studies; effectively results achieved by implementing the new protocol are the best ever noted.***

- ***In the second half year, the EURL-AP Proficiency Test Microscopy 2011 was prepared and organised. This test allowed NRLs as well as some third country participants to test their skills in the microscopic detection of PAPs. Seven types of blind samples were prepared with focus on PAP detection in fats (according to the dedicated protocol described under point 9 of Annex VI of EC Regulation EC/152/2009) and detection of hydrolysed feather meal. The homogeneity study was realised and showed samples fit for the study***

***purpose. As announced in September 2011, on the 7th of November samples were sent to the participants. Results had to be delivered by 25 November. Compilation and analysis of the results started in December and the writing of the report will be continued begin of 2012. All NRLs will receive the report before the next coming 2012 workshop in Berlin.***

3.3 Audit NRLs, coordinate training on methods of analysis and assist staff from NRLs if comparative testing reveals limited experience. Up to 3 European missions/year are foreseen in support to DG SANCO and/or EURL-AP activities. (1 p/m)

To help to develop, extend and keep in the NRLs the highest standard of technical skill and quality management under accreditation on analytical methods for detection, quantification and identification of animal proteins in feed ingredients and in feedingstuffs. (8 p/m) (*Note: This task has been merged with task 2.4.*)

*3.3.1 Definition of the needs of the NRLs.*

*3.3.2 Provide the requested help to the NRLs.*

*3.3.3 Preparation of syllabi including all the information needed for an appropriate detection of processed animal proteins in feedingstuffs.*

- ***No on-site visit was organised during in the 2011 period linked to underperformance for the NRLs.***

- ***A NRL argued during the 5th workshop that their laboratory found some terrestrial bones in the ILS2010 blank sample. Based on a respective agreement, they provided to the EURL-AP their permanent slides, where terrestrial bones were detected. The EURL-AP team looked at these slides and terrestrial particles were effectively found, nevertheless a good explanation of their presence was noted after looking meticulously at the NRL results (weight of the sediments): the NRL had inverted results and numbers from the blank sample and the previous one which effectively contained terrestrial***

***bones. The EURL-AP as well as the NRL considered the problem as closed.***

- ***Two NRLs were underperforming for the ILS2010. The underperfomance from the NRL1 was easily solved and the case closed; it was related to an abnormally high number of slides observations. The causes for the underperformance of NRL2 were investigated by the NRL2 which delivered acceptable explanations. With regards to the efforts made the EURL-AP considered the case was closed too.***

### 3.4 Organization of an interlaboratory study for GTH (with the collaboration of JRC-IRMM). (8 p/m)

- ***Concerning the preparation by the Analytical Chemistry Laboratory (CRA-W) of the samples necessary for the "Proficiency Test" organized by the EURL-AP, a first choice had to be made as for the spiking method of the samples. A study of the MBM spiking in mass was started by respecting the principle of the protocol used in industry (with and without dilution in a suitable organic solvent). The analysis of the samples obtained with this protocol is under development.***

- ***Due to the difficulty to obtain an homogeneous mass spiking on a laboratory scale, the choice of the samples preparation was made on an individual spiking: to a mass of known MBM (of which the content of fat contents is determined) a given volume of a GTH solution is added, the added volume is calculated to obtain a concentration well defined in the fat contents. For each concentration, 35 samples were prepared. A first study of homogeneity was carried out on 10 replicate samples taken randomly of each batch. The analysis of the results showed a rate of recovery lower than 90% and a great variability between samples (IRMM). Currently, the checking of the protocols continues in order to understand this variability and the rates of recovery obtained. This checking is necessary before sending the samples to the other participants of the ring trials.***

## 4 Development of analytical methods and tools. (42 p/m)

4.1 Contribute to the development of new methods of analysis and improvement of existing methods of analysis. (14p/m)

*4.1.1 Establishment/maintain of contact with the laboratory in charge of the development in order to be frequently informed about the progress of their development.*

*4.1.2 Definition of the potential support of the EURL-AP to these initiatives.*

*4.1.3 Establishment of the needs in the development of methods.*

*4.1.4 Evaluation of immunoassays proposed for the detection of PAP. (4 p/m)*

*4.1.5 Harmonization of analytical methods for determining insoluble impurities in rendered animal fat ('tallow') (Methods ISO 663 and AOCS Ca 3a-46). (7 p/m)*

*Note: This task refer to the request made by DG-Sanco in the letter sent on 28/07/2010 (Ref SANCO/D1/MK/(2010)/D).*

- ***Contacts with JRC-IRMM Reference materials Unit (Geel, Belgium): Full implementation of the PCR methods in the NRL network requires the use of a calibration by means of plasmids bearing the appropriate targets and provided in known amount to serve as reference material. This determination of the correct amount can only be done approximately now while it might be possible to get very precise figures with an uncertainty of measurement through use of digital PCR. As such a device is rather expensive the best way is to contact another laboratory equipped with it and that would be willing to collaborate. JRC-IRMM has such equipment. Contacts were established to that purpose already in May 2010 but it is only during May 2011 that the EURL-AP was officially authorized to have access to these facilities from September 2011 on. The 9th of September, a meeting was held at the JRC-IRMM (Geel, Belgium) with Dr Corbisier and the first tests started on the 27th of September. The unexpected presence of ruminant targets in one of the***

***reagents dedicated to digital PCR delayed the results until the 16th of November. This possibility had however been anticipated by the EURL-AP because it is known that BSA (bovine serum albumin) is frequently used in PCR mixes and in fact will only interfere if one is looking for bovine or ruminant DNA targets because trace amounts of DNA is still present. Therefore an alternative approach had been designed by the EURL-AP. In parallel however and thanks to the help of the European Technical Support of Fluidgim that developed the digital PCR system located at JRC-IRMM, it was possible to substitute the interfering reagent in question by another one free of ruminant DNA but that was not yet been used in digital PCR. The EURL-AP alternative approach which needs a special PCR target was also successful but use of the DNA free reagent was the preferred way to undertake the required measurements. By performing both analyses (with the alternative target compatible with the classical digital PCR reagent and the normal target with the DNA free reagent) evidence was gained that the new reagent proposed by Fluidigm was fit for digital PCR because initially it had not been set up for that purpose. The performed experiments allowed preparing a batch of reliable calibrants that will be used for the validation study of the PCR method of detection of ruminant DNA but also that will be provided to the NRLs for their first PCR proficiency test (2 actions that are planned in the first half year of 2012).***

- ***<u>Contacts with TNO Triskelion for assessment of their PCR methods (Zeist, The Netherlands)</u>: Here too contacts were established since some time with TNO Triskelion (formerly TNO), for assessment of the PCR techniques they developed, especially the one for ruminant. We received all technical information with respect to this technique at the beginning of March 2011. Assessment started immediately and will also provide the opportunity to check which acceptance criteria are crucial parameters to put as guidelines for PCR techniques. The full report on the assessment is not yet finalised but obtained performance of the method showed it could be considered for a full***

*validation through an interlaboratory study. The method was assessed for its specificity and its sensitivity (absolute and practical limit of detection) and competition effects that might influence these two latter properties were considered too. We found some aspecificities with sea mammals but it was not considered to be a major drawback. Another difficulty appeared linked to the fact (of which TNO Triskelion was totally unaware) that the target is present in very high copy number per cell (more than 100 000 copies). This requires some adaptations of validation protocols and the design of guidelines for acceptance parameters (this also clearly shows that merely transposing some of the guidelines that do exist for quantification of GMO's is not enough, there a lot of other new things to consider). The robustness of the test was checked and a precise protocol was outlined for setting a cut-off as this was the major issue missing in the TNO method to make it transferable. To that purpose, the ruminant targets (obtained by PCR done on bovine, ovine and caprine material) were cloned in a plasmid. However this last work had to be done again as it appeared in mid-June that one of the ordered primers had one sequence difference with what it ought to be. New plasmids were produced and can now be used as reliable calibrants to be measured precisely with the digital PCR technology in the beginning of 2012. With the preparation of the samples, it was the last step of the organisation of the validation study.*

*The Pig PCR method as well as the "poultry" PCR method of TNO Triskelion were also provided to the EURL-AP in October. Poultry has been put here between hyphens because TNO Triskelion does not know exactly to what range exactly of bird species the target is applicable (it is larger than chicken). These methods were considered on the same level as the PCR methods present in the NRLs and giving promising results during the PCR interlaboratory study conducted by the EURL-AP in February 2011 (see 4.3.) for further evaluation.*

- *Contacts with RIKILT and CCL about an interlaboratory study with two*

*immunochemical methods (MELISA-TEK and ReVeal) for detection of ruminant PAPs in non-ruminant PAPs (Gembloux, Belgium, 30th of November): Dr Leo van Raamsdonk (RIKILT) and Dr Rob Margry (CCL) presented the improvements obtained mainly with the MELISA-TEK kit since the last evaluation by the EURL-AP as well as the scope, the aim and the design of the interlaboratory study. The EURL-AP represented by Dr Gilbert Berben and Dr Olivier Fumière gave some advices on different points of the initiative and asked to participate to the study. Dr Leo van Raamsdonk asked if it was possible to integrate the immuno-assay method(s) in the annex VI if their validation was successful. This request was put in the minutes that were transmitted to DG-Sanco.*

- *<u>Contacts with JRC-IRMM Feed Quality Unit (Geel, Belgium)</u>: as previously there were several very fruitful contacts with the unit of Christoph von Holst. EURL-AP was for instance asked to comment a draft of a manuscript in which the full validation of a screening method is considered. The method developed here takes into consideration what EURL-AP had done for validation of the cut-off and the validation of the bovine PCR method in SAFEED-PAP but it extends the concept and provides general rules on how it should be done.*

- *During the first half 2011, the EURL-AP has received a total of 33 fat samples from EFPRA (European Fat Processors and Renderers Association). During this same period and during the end of 2010, the EURL-AP was recompiling the methods ISO 663 and AOCS Ca 3a-46. During the first half 2011 period, the EURL-AP has meticulously compared the protocols and looked for the necessary complementary laboratory devices and equipment needed. Analyses of the methods revealed that many optional parameters possibly influencing the results were included in the methods. Statistical analysis of those parameters will require the preparation of an experimental design. After having defined an experimental design, the EURL-AP started during the second half year of 2011 implementing ISO 663 method and testing its*

***multiple parameters. Results indicated that there were no significant differences between the two solvents proposed. Significant differences were noted among the types of filters. The EURL-AP team is at writing time focussing on the sub-sampling method and its homogeneity which seems to be crucial but problematic related to the amount of insoluble impurities that can be recovered from fats. Fats are actually sampled at production sites under melted state, before solidifying the insoluble impurities settle to the bottom of the vial. This results in a large heterogeneity of the fat. The method of sub-sampling does not consider this aspect but is accounting for a variability of the weight of insoluble impurities being recovered. The work will be continued in 2012 as well as the implementation of AOCS Ca 3a-46.***

- ***At the time of the method implementation of the GTH in category III livestock products (MBM), developed by IRMM (Geel), several parameters were studied during the second half-year 2011 by the Analytical Chemistry Laboratory of CRA-W before starting the analysis of the samples of the RT2008 provided by IRMM.***

  ***Among these parameters, a study of the fat extraction kinetics was carried out by using two protocols of extraction (Soxhlet and its automated version, Soxtec) associated with two organic solvents (hexane and petroleum ether). This study highlighted the variability of the Soxtec compared to the quantity of fat contents to be extracted. The extractions appeared to be rather incomplete and very variable. The possibility of reducing the time of the Soxhlet extraction using hexane as solvent was also studied. Indeed, after two hours of extraction, the recovery rates achieved were higher than 97%. This time reduction of extraction must however be confirmed and refined in order to avoid any selective extraction between the GTH and the fat contents present.***

  ***The second studied parameter related to the purification on SPE cartridges of the fat extract : comparison of three different cartridges and five matrices (butter, lard, olive oil, fat extracted from milk and fat extracted from MBM).***

***On the basis of the comparison between the fatty-acids profiles obtained with and without SPE purification, amino-bond $NH_2$ cartridges were chosen.***

***To conclude the study of these two parameters, the chromatographic (GC) and spectrometry (MS) methods were optimized on the equipment available in the laboratory (Agilent GC 6890 coupled to an Agilent mass spectrometer).***

***Following the optimization of the three stages of analysis (extraction, purification and chromatography), the CRA-W laboratory carried out the analysis of the samples of the Ring Trial organized in 2008 by the IRMM during the validation of the method. On the basis of result obtained and delivered to the IRMM, the method could be in-house validated.***

***The implementation of the analytical method took delay because of the setting in conformity of the hoods of laboratory. This transformation, initially envisaged in January 2012 was advanced in November 2011 and lasted nearly six weeks, preventing any work in the laboratory. Indeed, all handling relating to the extraction of the fat contents and its purification on cartridge SPE can be done only under hood, in order to preserve the health of the staff.***

***As of the end of the works, the team of the laboratory took again its activities, privileging the analyses relating to the samples of the proficiency test, organized by the EURL-AP. During the analysis of these samples, an important variability appeared between samples (individually spiked). Moreover, the rates of recovery turned in the neighbourhoods from the 85%. Before launching the proficiency test, it is essential to understand these results. The necessary investigations thus delayed the launching of the proficiency test***

## 4.2 Contribute to the development of complementary analytical methods necessary to assure the correct implementation of official methods and explorative or alternative methods. (3 p/m)

### *4.2.1 Test of methods for the detection of blood products*

4.3 Coordination of evaluation studies on alternative methods. As soon as they become available, methods specifically detecting ruminant, pig or poultry proteins should be evaluated. (18 p/m)

*4.3.1 Organisation of interlaboratory studies based on alternative tests (PCR, Immunology) developed by NRLs or by companies.*

*4.3.2 On the basis of former interlaboratory studies regarding PCR methods, define the strategy for the optimum implementation in the NRL*

*4.3.3 Preparation of EURL-AP protocol at the destination of the NRL for the implementation of the PCR methods*

*4.3.4 Organisation of the transfer of validated PCR methods to the NRLs network – training courses and manuals will be prepared during 2011*

*4.3.5 Production and verification of PCR kits for the NRLs*

- ***Following the results of the EURL-AP inquiry about the status of PCR in the NRLs conducted in 2010, an interlaboratory study was organized between January (official announcement) and March 2011 (results return). Its aims were to evaluate the potential of PCR targets present in the NRLs for the detection of PAPs (> 50 targets) and to identify assays that would be of interest for a future validation. Seventeen targets gave interesting results to be considered by the EURL-AP for further investigations on their fitness for the detection of PAPs. Nevertheless, a majority of the assays is not fit for the purpose or is not sensitive enough to be used as such in routine analysis.***

  ***Beginning of September, the EURL-AP asked to the NRLs those protocols identified as promising methods in order to submit them to an in-depth evaluation. In addition, methods were also provided by TNO Triskelion bv and by the Universidad Complutense de Madrid.***

- ***In between, the EURL-AP started the evaluation of a TNO Triskelion PCR protocol for the detection of ruminant DNA in feed in view of a possible validation study organised by the EURL-AP. The first results were very promising and demonstrated enough the potential of the assay to go to a***

***validation study. The invitations as well as Non Disclosure Agreements requested by TNO Triskelion were officially sent to different experienced participants approved by TNO Triskelion the 21st of December. The process is progressing according to the planned time schedule aiming to send the material of the study by the end of January 2012.***

- ***In order to check correctly the specificity of new PCR targets, the EURL-AP set up a DNA sample bank. When possible, the DNAs were extracted from blood samples in order to be sure of their purity. For the other species, the DNAs were extracted from meat samples. More than forty animal DNAs covering ruminant (cattle, sheep, goat, stag and roe deer), non-ruminant terrestrial mammalians (human, pig, donkey, horse, rabbit, hare and rat), birds (chicken, guinea fowl, turkey, duck, pheasant, pigeon and ostrich), sea mammals (6 species), fishes (20 species) and crab are gathered***.

4.4 Performing EURL-AP available methods or adapting them on outbreak material to make them available for the NRLs network. (2 p/m)

4.5 Extension of the samples bank with a special focus on specific animal material (e.g. sea mammals, rodents). Test, packaging and storage of the new samples as well as production of microscopic image representative of the particles making up the samples collected and selected to be included in the EURL-AP samples bank. (5 p/m)

*4.5.1 Establishment of the specification for the EURL-AP samples bank.*

*4.5.2 List of the priority needs regarding the materials to include in the samples bank.*

*4.5.3 Maintenance of informatics tools for the appropriate management of the samples.*

*4.5.4 Collection/production of samples of animal origin.*

*4.5.5 Test of the samples collected.*

*4.5.6 Storing of the samples and maintenance of the samples bank.*

## 5 Workshops/trainings. (8 p/m)

5.1 Provide specific workshop for the benefit of NRLs for the correct application of the method described in the Annex VI of the 152/2009/EC Commission regulation to detect animal proteins in feed (Classical microscopy) and any new development or regulation related to the detection, identification and quantification of animal proteins in feed. (8 p/m)

*5.1.1* Light microscopy training

*5.1.2 PCR training*

- ***During the first period of 2011, three training sessions on the microscopic method were organised at the EURL-AP facilities. From the 19th to 21st January, one representative from Denmark, Belgium, Lithuania and Poland participated in the training sessions. From the 23rd to 25th February NRL representatives from Portugal, Romania and Germany. The third session was held from the 28th to 30th of June, where representatives from Austria, Ireland and Czech Republic were present. In these training sessions, practical and theoretical content is included, didactical material as books and manuals are also commented. A syllabus including all the lectures and the agenda is given to the participants. Reference sample material as well as permanent slides made during the training are given to the participants for further didactic and reference material at their labs.***

  ***As already mentioned at point 2.4, the PCR training program continued with a new session in February gathering five new NRLs. Two other training sessions were organised in September (13-15 September) and in October (4-6 October) with for each session four participants invited on a voluntary basis. After five training sessions, a total of 19 NRLs already came in the EURL-AP facilities for a PCR training. The remaining NRLs (Denmark, Germany, Italy, Luxemburg, Sweden, The Netherlands and United Kingdom) are sufficiently proficient with PCR and did not consider it necessary to attend the EURL-AP training on PCR. For any training session, participants are now asked to fill an evaluation form***

***including aspects as content and organisation of the training. The EURL-AP team is very satisfied with the results of this evaluation. Assistance certificates were delivered at the end of the sessions to each participant.***

5.2 Provide specific workshop of experts from candidate Member States for the correct application of the 152/2009/EC directive to detect animal proteins in feed (Classical microscopy) and any new directive linked to the detection, identification and quantification of animal proteins in feed. (0 p/m)

5.3 Provide training through dissemination tools like CD's or DVD's. Development of analytical support and libraries for the training and the maintenance of the skill of laboratories performing classical microscopy or other validated method. (0 p/m)

- ***As already mentioned at the point 2.4, a DVD on the PCR was produced during summer and sent to all the NRLs at the beginning of autumn.***