EVALUATION OF THE NEOGEN IMMUNOASSAY « *REVEAL® FOR RUMINANT* » FOR THE DETECTION OF RUMINANT PROTEINS IN PROCESSED ANIMAL PROTEINS

ADDENDUM :

PRELIMINARY TESTS WITH THE « *MELISA-TEK™* » RUMINANT KIT FROM ELISA TECHNOLOGIES, INC., GAINESVILLE, FL, USA

OLIVIER FUMIÈRE, GILBERT BERBEN, VINCENT BAETEN

Walloon Agricultural Research Centre,
CRA-W Gembloux, Belgium
Community Reference Laboratory for Animal Proteins in Feedingstuffs,
CRL-AP Gembloux, Belgium

Final version – October 2008

ISBN 978-2-87286-063-0
Dépôt legal D/2008/1463/5

Edité par :
Centre wallon de Recherches agronomiques
Service communication
Rue de Liroux, 9
5030 Gembloux (Belgique)
cra@cra.wallonie.be
http://cra.wallonie.be

TABLE OF CONTENTS

1. INTRODUCTION

The outbreak of bovine spongiform encephalopathy (BSE) urged the European Union to take several decisions in order to avoid the transmission of its causal agent through the food chain. At present, unless exceptions for fish meal, processed animal proteins (PAPs) including meat and bone meals are banished from use as feed ingredients for all farmed animals. Moreover, the use of PAPs is controlled within the European Union through several regulations which on one hand prohibit explicitly the feeding of mammalian PAPs to ruminants (Regulation EC 999/2001), the feeding of animals with proteins from the same species (Regulation EC 1774/2002) and on the other hand establish 3 categories of animal by-products (ABP) reflecting different safety levels. In consequence, only material from category 3 which comprises material fit for human consumption can be used to feed farm animals.

For the moment, classical optical microscopy is the only official method for PAPs detection in compound feeds or in their ingredients in the European Union and there is a tremendous need for validated techniques able to detect routinely PAPs as well as to identify their origin at the species level before a reappraisal of the total MBM ban. Among the techniques to aim this goal, immunoassays based tests are of interest. The ***"ReVeal® for Ruminant"*** kits (Neogen Corporation, Lansing, MI, USA) were already studied several times. Two different tests are available : ***"ReVeal® for Ruminant in MBM"*** is an assay used for the qualitative analysis of beef and sheep MBM in non-ruminant protein meal. According to the user manual of the manufacturer, the test also reacts with by-product material from goats but it does not detect material from other ruminants such as deer or elk. ***"ReVeal® for Ruminant in Feed"*** is an assay to use for the qualitative analysis of ruminant skeletal muscle protein in compound feeds and feed ingredients. As declared by Neogen® Corporation, the test is not intended for use with meat and bone meals.

In 2006, based on a risk assessment study conducted by Det Norsk Veritas Ltd. (DNV consulting, 2006), EFPRA proposed the re-entry of certain PAPs for use in Feeds (EFPRA, 2006) respecting the intra-species ban laid down in the ABP Regulation (Regulation EC 1774/2002). More recently, considering that efficient control tools may not yet be in place and that it would be difficult to organise in practice its first proposal, EFPRA requested that DG-Sanco gives serious consideration to the use of non-ruminant PAPs in Feeds for Aquatic Species (Aqua-feeds) (Woodgate, 2007 a). Moreover, based on the results of studies conducted by the CCL Nutricontrol (Veghel, the Netherlands) (van den Hoven, 2007; Vaessen, 2007 a & b), EFPRA proposed to use the ***"ReVeal® for Ruminant in Feed"*** even as screening method for the detection of ruminant PAPs in PAPs (Woodgate, 2007 b).

Due to the performances of the method, a tolerance level of 1% (w/w) of ruminant material would also be introduced.

The purposes of the present document are : i) to summarize the data on the ***ReVeal® for Ruminant*** kits obtained from studies conducted by different laboratories and/or published in literature ; ii) to check if the ***"ReVeal® for Ruminant in Feed"*** is fit for the purpose outlined by CCL because this assay is not recommended by the manufacturer for the analysis of PAPs. To that end the performances of the ***"ReVeal® for Ruminant in Feed"*** and of the ***"ReVeal® for Ruminant in MBM"*** assays for the purpose of the detection of ruminant material in PAPs are compared and assessed ; and iii) to point out the possible risks of false results with a major focus on false negative results as it is proposed to use the assay as a first line screening technique. This means that positive results would be further analysed while for negative outcomes the products will be considered as correct.

2. PRINCIPLE OF THE TESTS

The ***"ReVeal® for Ruminant in MBM"*** and the ***"ReVeal® for Ruminant in Feed"*** tests are single-step lateral flow immuno-chromatographic assays performed on strips also called dipsticks (Figures 1 and 2).

FIGURE 1 : REVEAL® RUMINANT IN MBM STRIP TEST

FIGURE 2 : REVEAL® RUMINANT IN FEED STRIP TEST

The proteins are extracted thanks to a special buffer provided with the kit. This extract is wicked through a reagent zone, which contains antibodies specific for an heat stable epitope of the ruminant muscle protein Troponin I. These antibodies are conjugated to coloured particles (colloïdal gold). If ruminant by-product is present, it will be captured by the conjugated antibodies. The antibody-antigen complex is then wicked onto a membrane up to a test line zone where another antibody specific for the ruminant muscle protein is spotted. This zone captures the protein in a sandwich complex allowing the gold particles to concentrate and form a visible line (**Test line** - Figures 3 and 4). If no ruminant by-product is present, no line will form at that level of the dipstick. The membrane also contains a control zone where an immune complex present in the reagent zone captures any antibody, forming a visible line. The **Control line** (Figures 3 and 4) should always be formed regardless of the presence of ruminant by-product, thus ensuring the strip is working properly. If no control line appears the test must be considered as invalid. The possible results are illustrated at the Figure 7.

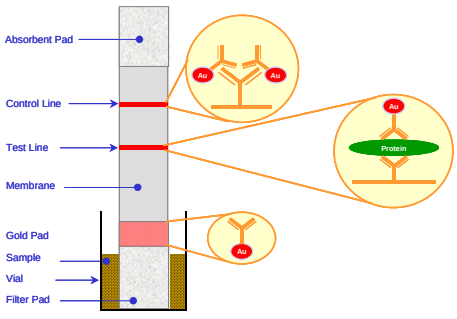


FIGURE 3 : GENERAL SCHEME OF A STRIP

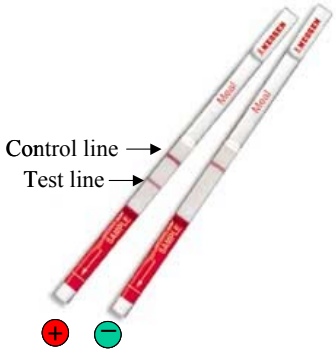


FIGURE 4 : STRIP OF A POSITIVE AND A NEGATIVE RESULT

The test strip provides results in approximately 15 minutes and the overall analytical process (weighing of the test portion, protein extraction and immuno-chromatography) is performed in ~ 45 minutes (Figure 5).

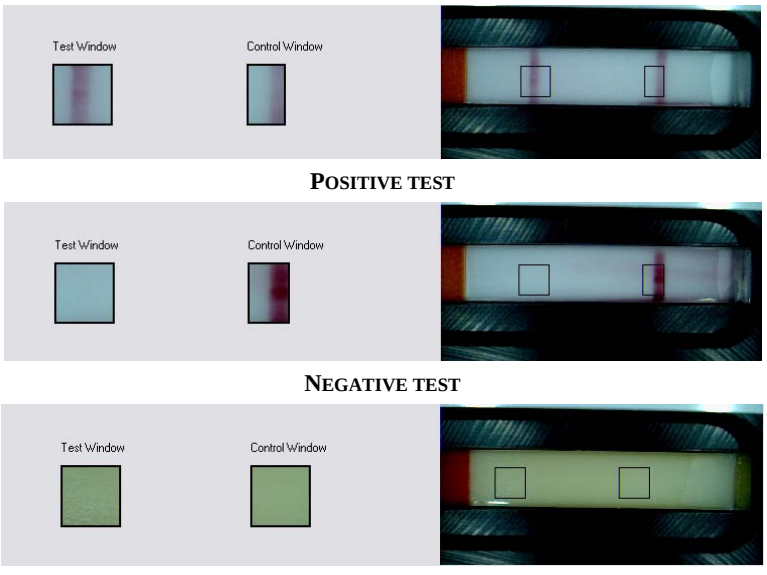
Weighing of the test portion | Addition of the extraction buffer | Heating in boiling water for 10 minutes | Transfer of the protein extract to a sample tube | Immunochromatography | Reading of the result

Overall process ~ 45 minutes/sample

FIGURE 5 : TEST PROCEDURE (PICTURES OF NEOGEN CORPORATION)

The analytical result of the dipstick can either be visually evaluated or more objectively assessed by using a dipstick reader, the ***"Accuscan Reader"*** from Neogen Corporation (Figure 6).

FIGURE 6 : ACCUSCAN READER FROM NEOGEN CORPORATION



POSITIVE TEST

NEGATIVE TEST

INVALID TEST

FIGURE 7 : POSSIBLE RESULTS OBTAINED WITH THE ACCUSCAN READER

3. STATUS OF THE KNOWLEDGE ON THE PERFORMANCES OF THE REVEAL® FOR RUMINANT ASSAYS

As stated before, the 2 available assays are dedicated to the analysis of different types of samples (feeds and feed ingredients at one side or animal meals at the other side). In former studies, the kits were always used as recommended by the manufacturer with a satisfying assessment of their potential.

In 2003, a study was conducted at the CRA-W in order to evaluate the potential of Neogen kits named at that moment ***"Agri-Screen for Ruminant in MBM"*** and ***"Agri-Screen for Ruminant in Feed"*** (Lafortune, 2003 ; Fumière *et al.*, 2004). The conclusions of that work and of additional evaluations were that the limits of detection announced by the manufacturer (*"Agri-Screen for Ruminant in MBM"* : 5 % of ruminant MBM in fish meal and 2 % of ruminant MBM in non-ruminant (pig and chicken) animal meal - *"Agri-Screen for Ruminant in Feed"* : 1 % of ruminant MBM in feeds or feed ingredients) were in general correct. The test is even able, in some cases, to detect lower ruminant contents (~ 0.25 %) in feeds and feed ingredients. Nevertheless, sometimes a lack of robustness of the method appeared : i) some bovine MBM heat treated at temperatures > 133°C were not detected ; ii) the detection of ruminant proteins in spiked pig meal was less efficient due to a masking effect of some yet unknown compounds from the pig meals ; iii) aspecificity was observed with pure beetpulp but never occurred with feeds containing beetpulp even at a 15% level ; iv) some raw materials commonly used in feeds gave invalid result (waffle meal, wheat and tapioca). Moreover, as in 2004 the ***Accuscan Reader*** was not yet available at CRA-W, the conclusion on the result of the test was subjective and dependent on the analyst, particularly for feed materials with low ruminant content giving very faint test lines (Fumière *et al.*, 2004).

In an intercomparison study conducted by the JRC-IRMM (Boix *et al*., 2004), the ***"Reveal® for Ruminant Feed Test System"*** was evaluated among three other commercially available immunoassays regarding their capability to detect MBM in feed at 0.1 % (w/w). The study revealed the potential of the dipstick method developed by Neogen for the intended purpose which was the detection of PAPs from ruminants in feed. As a follow-up to that study, the JRC-IRMM conducted, in 2006 a ruggedness study in order to establish the impact of various feed ingredients on the analytical results and to evaluate the transferability of the method from the laboratory that developed the test to another laboratory before conducting a full validation of the method through an interlaboratory study (Boix *et al*., 2006).

The commercially available test kit developed by Neogen ***"Reveal® for Ruminant Feed Test System"*** successfully passed the ruggedness test and the validation of the method by an intercomparison study at European level was recommended. The test showed a sufficient sensitivity at the level of 0.5 % ruminant PAPs but insufficient sensitivity when the samples of feed contained 0.1 % ruminant

PAPs. Some of the blank animal feed samples were wrongly classified as positive. The presence of animal fats from rendering industry might be a source of false positive results especially in pig feeds where this animal fat is frequently used. Some false positive results were also related to beetpulp or citruspulp used as ingredients in compound feeds. However, these "false" results do not represent any major problem when integrating the method in a global control system, applying the dipstick test mainly for screening purposes. Positive samples would then need to be tested by a confirmatory method.

A study was also published in 2005 by Myers *et al.* dealing with the performances of the ***Reveal® for Ruminant in Feed*** test. The results differed slightly from the ruggedness study conducted by IRMM. In this study, the ***Reveal*** test demonstrated a perfect selectivity and did not achieve a 0.1 % level of sensitivity. It must be mentioned that the results in this study did not take into account the problems observed elsewhere with ingredients such as beetpulp, citruspulp and fish meal and were obtained with American animal meals (i.e. rendered with a less stringent process compared to what is performed in Europe for mammalian PAPs).

Finally, the ***"ReVeal® for Ruminant in Feed"*** and the ***"ReVeal® for Ruminant in MBM"*** tests were also subjected to validation studies conducted by the AOAC Research Institute in 2004 and 2005 respectively. According to the certification marks, the sensitivity of the tests are : 1 % of ruminant material in feed for the ***"ReVeal® for Ruminant in Feed"*** (Certificate n° 010405 ; AOAC, 2004) and 1 - 2 % of ruminant material in MBM for the ***"ReVeal® for Ruminant in MBM"*** (Certificate n° 070501 ; AOAC, 2005).

An overview of the main features (sensitivity and drawbacks) obtained along several studies carried out on both types of assays are given in Tables 1 and 2.

TABLE 1 :
SENSITIVITY OF THE REVEAL® FOR RUMINANT IN MBM AND REVEAL® FOR RUMINANT IN FEED ASSAYS

Source	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Neogen Corporation	✓2-5% ruminant skeletal muscle protein in meat and bone meal.	✓1% of ruminant skeletal muscle protein in feed supplements and finished feeds.
CRA-W	✓2 % of ruminant MBM in non ruminant meat and bone meal. ✓5 % of ruminant MBM in fish meal.	✓1% of ruminant skeletal muscle protein in feed supplements and finished feeds.
AOAC	✓1-2% of ruminant material in MBM.	✓1% of ruminant material in feed
JRC-IRMM	Not tested	✓0.1-0.5% of ruminant PAP in feed.
CCL Nutricontrol		✓0.1-0.5% of ruminant PAP in feed. ✓0.1-0.2% of ruminant PAP in MBM.

TABLE 2 :
DRAWBACKS OF THE REVEAL® FOR RUMINANT IN MBM AND REVEAL® FOR RUMINANT IN FEED ASSAYS

Source	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
CRA-W	✓ False negative results with some pure cattle PAPs ✓ False negative results with some cattle PAPs heat treated > 133°C ; ✓ False negative results with pig meal adulterated with cattle meal ("Masking effect").	✓ False negative results on 2% ruminant in presence of alfalfa ; ✓ False positive results with beetpulp ; ✓ Inconclusive results with wheat and manioc meal.
JRC-IRMM	Not tested	✓ Possible false positive results in feeds when presence of beetpulp, citruspulp or fats.
CCL Nutricontrol	✓ False negative results with pure ruminant materials → Dilution should enable detection of ruminant tissue ; ✓ Test strip sometimes coloured by the sample extract → White line in the sample zone giving rise to a false positive result with the Accuscan Reader.	✓ Chicken material might give false positive results ;

4. SAMPLE PREPARATION AND ANALYSIS

The test materials used in this study were selected within the CRL-AP sample bank. They **are as much as possible representative of typical PAPs present in Europe but some PAPs from other origins (USA and Australia) were also incorporated in the study.** All the samples used were previously analysed at CRA-W by Real Time PCR using mitochondrial targets (Fumière *et al.*, 2006). For all the samples, the proteins were extracted once and tested with 2 dipsticks in order to prevent and evaluate any possible heterogeneity of the dipsticks. As much as possible, the samples were analysed with both kits (MBM and Feed) in order to evaluate the respective performances of the 2 versions of the kit. The results were read with the ***Accuscan Reader*** (Neogen Corporation) but were also examined visually. When a visual examination of the strip gave conflicting conclusions, it is mentioned with a remark in the tables. As it could interfere with the test, the pH of the extract of each sample was also estimated with the help of a pH indicator paper.

4.1. SPECIFICITY OF THE TEST : ANALYSIS OF PURE PAPS

The first step of the study was to evaluate the specificity of the test. Pure PAPs from different species (Cattle, Pig, Poultry, Fish) were analysed with both assays. Samples in which cattle DNA was detected by PCR were also tested.

TABLE 3 (1ST PART) :
ANALYSIS OF PURE CATTLE PAPS (PCR TESTED) - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/02/0410	1: Negative 2: Negative	1: Positive 2: Positive
DQ/02/0411	1: Negative 2: Negative	1: Positive 2: Negative
DQ/02/0862-02	NT **	1: Positive 2: Positive
DQ/02/1032	1: Positive 2: Positive	1: Positive 2: Positive
DQ/06/0959-17	1: Positive 2: Positive	1: Positive 2: Positive
DQ/07/0134-01	1: Negative 2: Negative	1: Positive 2: Positive
DQ/07/0134-01 (extract 10-fold diluted)	1: Positive 2: Positive	NT *

* NT : not tested as no dilution of the extract is needed to detect the presence of the target.
** NT : not tested. Too small amount of sample.

TABLE 3 (2ND PART) :
ANALYSIS OF PURE CATTLE PAPS (PCR TESTED) - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/07/0134-02	1: Negative 2: Negative	1: Positive 2: Positive
DQ/07/0134-02 (extract 10-fold diluted)	1: Positive 2: Positive	NT *
DQ/07/0134-03	1: Negative 2: Positive	1: Positive 2: Positive
DQ/07/0134-03 (extract 10-fold diluted)	1: Positive 2: Positive	NT *
DQ/07/0134-04	1: Positive 2: Positive	1: Positive 2: Positive
DQ/07/0134-04 (extract 10-fold diluted)	1: Positive 2: Positive	NT *

* NT : not tested as no dilution of the extract is needed to detect the presence of the target.

As already observed previously, the ***"ReVeal® for Ruminant in MBM"*** assay can fail to detect pure cattle PAPs. With some of these samples, a positive result has been obtained when a tenfold dilution of the protein extract with the kit extraction buffer was tested. The ***"ReVeal® for Ruminant in Feed"*** showed a better robustness and gave positive results without any dilution. The pH of the extracts were about 6.0 with the ***"ReVeal® for Ruminant in MBM"*** (on undiluted as well as on tenfold diluted extracts) and 6.5 with the ***"ReVeal® for Ruminant in Feed"*** extracts.

TABLE 4 (1ST PART) :
ANALYSIS OF PURE PIG PAPS (PCR TESTED) - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/02/1114-01	NT *	1: Negative 2: Negative
DQ/01/1114-03	NT *	1: Negative 2: Negative
DQ/07/0038-01	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/0094-04	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/0094-05	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/0134-09	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/0134-10	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/1089-19	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/1089-20	1: Negative 2: Negative	1: Negative 2: Negative

* NT : not tested. Too small amount of sample.

TABLE 4 (2ND PART) :
ANALYSIS OF PURE PIG PAPS (PCR TESTED) - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/07/1089-22	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/1089-23	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/1089-24	1: Negative 2: Negative	1: Positive 2: Positive
DQ/07/1089-25	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/1089-26	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/07/1089-27	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/07/1089-28	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/0339-04	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/07/0339-05	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/0099-02	1: Negative 2: Negative	1: Negative 2: Negative

* Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.

As also reported by CCL Nutricontrol, the dipstick can be coloured by the sample extract and give a clearer test line (see Figure 7 - page 26). In such a case, the ***Accuscan Reader*** declares the sample as positive even if it is visually evident that the sample is negative. All the remaining samples gave the expected result whatever the test used except one sample (DQ/07/1089-24) tested with ***"ReVeal® for Ruminant in Feed"*** giving a false positive result.

TABLE 5 :
ANALYSIS OF PURE POULTRY PAPS (PCR TESTED) - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/07/1089-03	1: Negative 2: Negative	1: Positive 2: Positive
DQ/07/1089-04	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/1089-06	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/1089-07	1: Negative 2: Negative	1: Positive 2: Positive
DQ/07/1089-10	1: Negative 2: Negative	1: Negative 2: Negative
DQ/07/0339-06	1: Negative 2: Negative	1: Negative 2: Negative

False positive results are observed when the ***"ReVeal® for Ruminant in Feed"*** kit is used for the analysis of poultry meals. The same samples analysed with the ***"ReVeal® for Ruminant in MBM"*** kit gave negative results as expected. The specificity of the ***"ReVeal® for Ruminant in Feed"*** test may fail giving rise to false positive results. One feather meal was also analysed and gave a negative result whatever the kit used (data not shown).

TABLE 6:
ANALYSIS OF PURE FISH MEALS (PCR TESTED) - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/02/0838	1: Negative 2: Negative	1: Negative 2: Negative
DQ/04/0220	1: Negative 2: Negative	1: Negative 2: Negative
DQ/04/0239	1: Negative 2: Negative	1: Negative 2: Negative
DQ/04/0335	1: Negative * 2: Negative	1: Negative 2: Negative
DQ/04/0484	1: Negative 2: Negative	1: Negative 2: Negative
DQ/05/0653-14	1: Negative 2: Negative	1: Negative 2: Negative
DQ/05/0653-15	1: Negative 2: Negative	1: Negative 2: Negative
DQ/05/0653-17	1: Negative 2: Negative	1: Negative 2: Negative
DQ/05/0653-18	1: Negative 2: Negative	1: Negative 2: Negative
DQ/05/0653-19	1: Negative 2: Negative	1: Negative 2: Negative
DQ/05/0653-20	1: Negative 2: Negative	1: Negative 2: Negative
DQ/05/0653-23	1: Negative 2: Negative	1: Negative 2: Negative

* Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.

All the samples gave the expected negative result whatever the assay used. The same problem of coloration of the dipstick as described with the pig PAPs was observed once (*) but, this time, a second measurement of the dipstick with the ***Accuscan Reader*** gave a negative result.

4.2. ANALYSIS OF PAPS CONTAINING CATTLE DNA DETECTED BY PCR

One of the main objections against the use of PCR is its high sensitivity able to detect traces of DNA with the hasty conclusion that the method does not fit for the

purpose. Different samples of PAPs where cattle DNA was detected by PCR were also analysed with the ***"ReVeal® for Ruminant"*** kits in order to compare the results of both methods.

TABLE 7 :
ANALYSIS OF PIG PAPS WITH CATTLE DNA DETECTED BY PCR - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/07/0213	1: Positive 2: Positive	1: Positive 2: Positive
DQ/07/1089-29	1: Negative 2: Negative	1: Negative 2: Negative

TABLE 8 :
ANALYSIS OF POULTRY PAPS WITH CATTLE DNA DETECTED BY PCR - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/07/1089-09	1: Negative 2: Negative	1: Positive 2: Positive
DQ/07/1089-11	1: Negative 2: Negative	1: Negative * 2: Negative *

* Conflicting results given by several successive reading with the Accuscan Reader.

TABLE 9 :
ANALYSIS OF FISH MEAL WITH CATTLE DNA DETECTED BY PCR - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Sample code	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/07/1089-14	1: Negative 2: Negative	1: Negative 2: Negative

The presence of cattle material in one sample of pig PAPs (DQ/07/0213) is clearly confirmed by positive results with both ReVeal tests (Table 7). On the contrary, the positive result obtained with the ***"ReVeal® for Ruminant in Feed"*** kit (Table 8) must be taken cautiously as aspecificity of the assay was observed with pure poultry PAPs (see Table 5). The negative results obtained with the remaining samples can not exclude the presence of bovine material in the samples as the limit of detection of the ***"ReVeal® for Ruminant"*** tests is probably higher than the CRA-W PCR method.

4.3. ESTIMATION OF THE LIMIT OF DETECTION OF THE TESTS : ANALYSIS OF ADULTERATED PAPS

The EFPRA proposal for the use of non-ruminant processed proteins in Aqua-feeds takes into account the limits of the ReVeal tests observed by the CCL and recommends the level of 1-2 % as a tolerance threshold for the presence of ruminant PAP in non-ruminant PAP. For that reason, the samples tested were adulterated keeping in mind this target level.

4.3.1. Pig PAPs adulterated with cattle PAPs

Mixes 1 and 2 were prepared with pig PAPs (ref. DQ/07/0134-09) heat treated at 141°C and adulterated with an American cattle PAPs (ref. DQ/06/0959-17) at levels of 1 and 2 % respectively. **Mixes 3 and 4** were made of a second pig PAPs (ref. DQ/07/0134-10) heat treated at 133°C with the same American cattle PAPs (ref. DQ/06/0959-17) added at levels of 1 and 2 % respectively. **Mixes 5 to 8** were constituted of 99 % of another pig PAPs (ref. DQ/07/1089-19) and 1 % of cattle PAPs (ref. DQ/07/0134-01 to -04) heated treated at 145°C, 141 °C, 137°C and 133°C respectively. **Mixes 9 to 12** contained 99 % of a fourth pig PAP (ref. DQ/07/1089-26) and 1 % of the same cattle PAPs (ref. DQ/07/0134-01 to -04).

TABLE 10 :
ANALYSIS OF PIG PAPS ADULTERATED WITH CATTLE PAPS AT LEVELS OF 1 OR 2 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 1 (99% Pig PAPs 141°C + 1% U.S .Cattle PAPs)	1: Negative * 2: Negative	1: Negative 2: Negative
Mix 2 (98 % Pig PAPs 141°C + 2% U.S. Cattle PAPs)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 3 (99% Pig PAPs 133°C + 1% U.S. Cattle PAPs)	1: Negative 2: Negative *	1: Negative 2: Negative
Mix 4 (98 % Pig PAPs 133°C + 2% U.S. Cattle PAPs)	1: Negative * 2: Negative	1: Negative 2: Negative
Mix 5 (99% Pig PAPs + 1% Cattle PAPs 145°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 6 (99% Pig PAPs + 1% Cattle PAPs 141°C)	1: Negative * 2: Negative	1: Positive 2: Positive
Mix 7 (99% Pig PAPs + 1% Cattle PAPs 137°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 8 (99% Pig PAPs + 1% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 9 (99% Pig PAPs + 1% Cattle PAPs 145°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 10 (99% Pig PAPs + 1% Cattle PAPs 141°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 11 (99% Pig PAPs + 1% Cattle PAPs 137°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 12 (99% Pig PAPs + 1% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Positive 2: Positive

* Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.

With the 2 pig PAPs heat treated at different temperatures, an adulteration at a level up to 2 % with the American cattle is not detected whatever the test used for **mixes 1 to 4**. Nevertheless, the ***"ReVeal® for Ruminant in Feed"*** assay is able to detect the presence of 1 % of European cattle PAPs in the other samples. Additional tests were then performed in order i) **to estimate the level of contamination needed**

to detect the presence of cattle PAPs in the pig PAPs and ii) **to reproduce the same effect with other pig and cattle PAPs**.

Mixes 13 to 22 were prepared with 2 pig PAPs (ref. DQ/07/0134-09 and -10) heat treated at 141°C and 133°C respectively and adulterated with American cattle PAPs (ref. DQ/06/0959-17) at levels of 5, 10, 20, 30 and 40 %. The mixes analysed with the test ***"ReVeal® for Ruminant in Feed"*** remains negative until a partial detection of a 30 % level of contamination and a full detection at **40 %**. For the **mixes 13 to 22**, we diluted the protein extract tenfold with the kit buffer but the test gave positive results only for the **mixes 17 (20 % of cattle PAPs) to 22 (50% of cattle PAPs)**.

TABLE 11 (1ST PART) :
ANALYSIS OF PIG PAPS ADULTERATED WITH CATTLE PAPS AT A LEVEL OF 5, 10, 20, 30, 40 AND 50 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 13 (95% Pig PAPs 133°C + 5% U.S. Cattle PAPs)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 13 Extract ten-fold diluted	NT *	1: Negative 2: Negative
Mix 14 (95% Pig PAPs 141°C + 5% U.S. Cattle PAPs)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 14 Extract ten-fold diluted	NT *	1: Negative 2: Negative
Mix 15 (90% Pig PAPs 133°C + 10% U.S. Cattle PAPs)	1: Negative 2: Negative **	1: Negative 2: Negative
Mix 15 Extract ten-fold diluted	NT *	1: Negative 2: Negative
Mix 16 (90% Pig PAPs 141°C + 10% U.S. Cattle PAPs)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 16 Extract ten-fold diluted	NT *	1: Negative 2: Negative
Mix 17 (80% Pig PAPs 133°C + 20% U.S. Cattle PAPs)	NT *	1: Negative 2: Negative
Mix 17 Extract ten-fold diluted	NT *	1: Positive 2: Positive
Mix 18 (80% Pig PAPs 141°C + 20% U.S. Cattle PAPs)	NT *	1: Negative 2: Negative
Mix 18 Extract ten-fold diluted	NT *	1: Positive 2: Positive
Mix 19 (70% Pig PAPs 133°C + 30% U.S Cattle PAPs)	NT *	1: Positive 2: Negative
Mix 19 Extract ten-fold diluted	NT *	1: Positive 2: Positive

** Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.
* NT: not tested.

TABLE 11 (2ND PART) :
ANALYSIS OF PIG PAPS ADULTERATED WITH CATTLE PAPS AT A LEVEL OF 5, 10, 20, 30, 40 AND 50 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 20 (70% Pig PAPs 141°C + 30% U.S. Cattle PAPs)	NT *	1: Negative 2: Negative
Mix 20 Extract ten-fold diluted	NT *	1: Positive 2: Positive
Mix 21 (60% Pig PAPs 141°C + 40% U.S. Cattle PAPs)	NT *	1: Positive 2: Positive
Mix 21 Extract ten-fold diluted	NT *	NT **
Mix 22 (50% Pig PAPs 141°C + 50% U.S. Cattle PAPs)	NT *	1: Positive 2: Positive
Mix 22 Extract ten-fold diluted	NT *	NT **

* NT : not tested.
** NT : not tested as no dilution of the extract is needed to detect the presence of the target.

Four additional mixes (**mixes 23 to 26**) were prepared with the pig PAPs heat treated at 141°C (DQ/07/134-09) adulterated with 4 other cattle PAPs (DQ/02/410, DQ/02/411, DQ/02/862-2 and DQ/02/1032) at a level of 10%. These 4 cattle PAPs come from different origins : the 2 first are from E.U., the third one comes from Australia, and the last one is an American one.

TABLE 12 :
ANALYSIS OF PIG PAPS ADULTERATED WITH CATTLE PAPS AT A LEVEL OF 10 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in Feed
Mix 23 Pig PAPs (DQ/07/0134-09) + 10% E.U. Cattle PAPs (DQ/02/0410)	1: Negative 2: Negative
Mix 23 Extract ten-fold diluted	1: Negative 2: Negative
Mix 24 Pig PAPs (DQ/07/0134-09) + 10% E.U. Cattle PAPs (DQ/02/0411)	1: Negative 2: Negative
Mix 24 Extract ten-fold diluted	1: Negative 2: Negative
Mix 25 Pig PAPs (DQ/07/0134-09) + 10% Australian Cattle PAPs (DQ/02/862-2)	1: Negative 2: Negative
Mix 25 Extract ten-fold diluted	1: Negative 2: Negative
Mix 26 Pig PAPs (DQ/07/0134-09) + 10% U.S. Cattle PAPs (DQ/02/1032)	1: Negative 2: Negative
Mix 26 Extract ten-fold diluted	1: Positive 2: Positive

With these 4 cattle PAPs, a contamination level of 10 % is not detected except with the **mix 26** but after dilution of the extract. These results indicate a susceptibility of the test to the process as the cattle PAPs used in the mixes are

treated more (European samples) or less (Australian and American samples) drastically compared to the minimal European legal requirements for heat treatment. Indeed, different DNA degradation status are confirmed by the PCR results obtained with these samples : huge degradation on samples DQ/02/0410 and DQ/02/0411 and low degradation on samples DQ/02/862-2 and DQ/02/1032.

Mixes 27 to 38 were prepared using 2 pig PAPs (ref. DQ/07/0134-09 and -10) adulterated at a level of 1 or 2% with cattle PAPs (ref. DQ/07/0134-01 to -04) also used in mixes of Table 10 but in combination with other pig PAPs.

TABLE 12 :
ANALYSIS OF PIG PAPS ADULTERATED WITH CATTLE PAPS AT A LEVEL OF 1 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 27 (99% Pig PAPs 141°C + 1% Cattle PAPs 145°C)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 28 (98% Pig PAPs 141°C + 2% Cattle PAPs 145°C)	NT *	1: Negative 2: Negative
Mix 29 (99% Pig PAPs 141°C + 1% Cattle PAPs 141°C)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 30 (98% Pig PAPs 141°C + 2% Cattle PAPs 141°C)	NT *	1: Negative 2: Negative
Mix 31 (99% Pig PAPs 141°C + 1% Cattle PAPs 137°C)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 32 (98% Pig PAPs 141°C + 2% Cattle PAPs 137°C)	NT *	1: Negative 2: Negative
Mix 33 (99% Pig PAPs 141°C + 1% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 34 (98% Pig PAPs 141°C + 2% Cattle PAPs 133°C)	1: Positive 2: Positive	1: Positive 2: Positive
Mix 35 (99% Pig PAPs 133°C + 1% Cattle PAPs 145°C)	1: Negative ** 2: Negative **	1: Negative 2: Negative
Mix 36 (99% Pig PAPs 133°C + 1% Cattle PAPs 141°C)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 37 (99% Pig PAPs 133°C + 1% Cattle PAPs 137°C)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 38 (99% Pig PAPs 133°C + 1% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Negative 2: Negative

* NT : not tested. Too small amount of sample.
** Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.

These results indicate that the pig PAPs tested can mask the presence at the level of 1 % of another cattle PAPs than the ones tested previously (Table 10). In these cases, the false negative results are clearly due to a masking effect of the pig PAPs.

In addition, **mixes 39 to 46** were prepared with 2 pig PAPs heat treated at 133°C and 141°C (ref. DQ/02/1114-01 and -03) adulterated with other cattle PAPs (ref. DQ/07/0134-03 and -04) at levels of 1 and 2 %. **Mixes 47 and 48** contain 99 % of the same pig PAPs and 1 % of American cattle PAPs (ref. DQ/06/0959-17). At

last, **mixes 49 and 50** were made with 99 % of 2 other pig PAPs (ref. DQ/07/1089-19 and -26) and 1 % of American cattle PAPs (ref. DQ/06/0959-17).

TABLE 13 :
ANALYSIS OF PIG PAPS ADULTERATED WITH CATTLE PAPS AT LEVELS OF 1 AND 2 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 39 (98% Pig PAPs 141°C + 2% Cattle PAPs 137°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 40 (98% Pig PAPs 141°C + 2% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 41 (98% Pig PAPs 133°C + 2% Cattle PAPs 137°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 42 (98% Pig PAPs 133°C + 2% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 43 (99% Pig PAPs 141°C + 1% Cattle PAPs 137°C)	NT *	1: Negative 2: Negative
Mix 44 (99% Pig PAPs 141°C + 1% Cattle PAPs 133°C)	NT *	1: Positive 2: Negative
Mix 45 (99% Pig PAPs 133°C + 1% Cattle PAPs 137°C)	NT *	1: Positive 2: Positive
Mix 46 (99% Pig PAPs 133°C + 1% Cattle PAPs 133°C)	NT *	1: Positive 2: Positive
Mix 47 (99% Pig PAPs 141°C + 1% U.S. Cattle PAPs)	NT *	1: Negative 2: Negative
Mix 48 (99% Pig PAPs 133°C + 1% U.S. Cattle PAPs)	NT *	1: Negative 2: Negative
Mix 49 (99% Pig PAPs 141°C + 1% U.S. Cattle PAPs)	1: Negative 2: Negative	1: Negative 2: Negative
Mix 50 (99% Pig PAPs 133°C + 1% U.S. Cattle PAPs)	1: Negative 2: Negative	1: Negative 2: Negative

* NT: not tested as the test was not able to detect the presence of the target at the level of 2 %.

Here again, the different mixes tested gave false negative results at the level of **1 %**. The phenomenon is not limited to one pig PAPs and one cattle PAPs. Five pig PAPs (ref. DQ/02/1114-03, DQ/07/0134-09 and -10, DQ/07/1089-19 and -26) and five cattle PAPs (ref. DQ/06/0959-17 and DQ/07/0134-01 to -04) are concerned.

4.3.2. Poultry PAPs adulterated with cattle PAPs

Six mixes (**Mixes 51 to 56**) were prepared using 99 % of the same poultry PAPs (ref. DQ/07/1089-06) with 1 % of cattle PAPs (ref. DQ/07/0134-01 to -04) heat treated at 145°C, 141 °C, 137°C and 133°C respectively and with the American cattle PAPs DQ/06/0959-17 at levels of 1 and 2 %.

TABLE 14 :
ANALYSIS OF POULTRY PAPS ADULTERATED WITH CATTLE PAPS AT A LEVEL OF 1 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 51 (99% Poultry PAPs + 1% Cattle PAPs 145°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 52 (99% Poultry PAPs + 1% Cattle PAPs 141°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 53 (99% Poultry PAPs + 1% Cattle PAPs 137°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 54 (99% Poultry PAPs + 1% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 55 (99% Poultry PAPs + 1% U.S. Cattle PAPs)	NT *	1: Negative 2: Negative
Mix 56 (98% Poultry PAPs + 2% U.S. Cattle PAPs)	NT *	1: Negative 2: Negative

* NT: not tested.

The sensitivity of the test is improved by using the ***"ReVeal® for Ruminant in Feed"*** assay which is able to detect 1 % of cattle PAPs in poultry PAPs except with the cattle PAPs DQ/06/0959-17. These results again illustrate that some of the false negative results are due to the lower heat treatment of the cattle PAPs (DQ/06/0959-17 not detected at 1-2 % level in any other terrestrial PAPs in which it was put).

4.3.3. Fish meals adulterated with cattle PAPs

Mixes 57 and 58 were prepared with a fish meal (ref. DQ/05/0653-14) to which cattle PAPs (ref. DQ/06/0959-17) was added at the levels of 2 and 1 % respectively. **Mixes 59 and 60** were made of a second fish meal (ref. DQ/02/0838) with the same cattle PAPs (ref. DQ/06/0959-17) added at levels of 2 and 1 % respectively. **Mixes 61 to 64** contain the same fish meal as mixes 57 and 58 (ref. DQ/05/0653-14) but adulterated with 1 % of cattle PAPs (ref. DQ/07/0134-01 to -04) heated treated at 145°C, 141°C, 137°C and 133°C respectively. **Mixes 65 to 68** were made of a third fish meal (ref. DQ/07/1089-14) adulterated with 1 % of the same cattle PAPs as mixes 61 to 64 (ref. DQ/07/0134-01 to -04). **Mix 69** was prepared with the same fish meal (ref. DQ/07/1089-14) adulterated with 1 % of the cattle PAPs used in mixes 57 and 58 (ref. DQ/06/0959-17).

TABLE 15 :
ANALYSIS OF FISH MEALS ADULTERATED WITH CATTLE PAPS AT LEVELS OF 1 OR 2 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 57 (98% Fish meal 1 + 2% U.S. Cattle PAPs)	1: Negative 2: Negative *	1: Positive 2: Positive
Mix 58 (99% Fish meal 1 + 1% U.S. Cattle PAPs)	1: Negative * 2: Negative *	1: Negative 2: Positive
Mix 59 (98% Fish meal 2 + 2% U.S. Cattle PAPs)	1: Negative * 2: Negative	1: Positive 2: Positive
Mix 60 (99% Fish meal 2 + 1% U.S. Cattle PAPs)	1: Negative * 2: Negative *	1: Negative 2: Negative
Mix 61 (99% Fish meal 1 + 1% Cattle PAPs 145°C)	1: Negative 2: Negative *	1: Positive 2: Positive
Mix 62 (99% Fish meal 1 + 1% Cattle PAPs 141°C)	1: Negative 2: Negative *	1: Positive 2: Positive
Mix 63 (99% Fish meal 1 + 1% Cattle PAPs 137°C)	1: Negative * 2: Negative	1: Positive 2: Positive
Mix 64 (99% Fish meal 1 + 1% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 65 (99% Fish meal 3 + 1% Cattle PAPs 145°C)	1: Negative * 2: Negative *	1: Positive 2: Positive
Mix 66 (99% Fish meal 3 + 1% Cattle PAPs 141°C)	1: Negative * 2: Negative	1: Positive 2: Positive
Mix 67 (99% Fish meal 3 + 1% Cattle PAPs 137°C)	1: Negative 2: Negative *	1: Positive 2: Positive
Mix 68 (99% Fish meal 3 + 1% Cattle PAPs 133°C)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 69 (99% Fish meal 3 + 1% U.S. Cattle PAPs)	1: Negative * 2: Negative	1: Positive 2: Positive

* Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.

With the ***"ReVeal® for Ruminant in Feed"*** assay, a level of **1 %** of contamination with cattle PAPs in fish meal was detected in all the mixes tested, except the American PAPs (Mix 60). Here again, the use of the ***"ReVeal® for Ruminant in Feed"*** improves the sensitivity of the test. It has to be also pointed out that the reading of the ***"ReVeal® for Ruminant in MBM"*** dipsticks by the Accuscan Reader gave frequently conflicting results with their visual examination.

4.3.4. Fish meals adulterated with cattle and pig PAPs

In order to see if the potential masking effect of cattle PAPs by pig PAPs could lead to false negative results in the analysis of fish meals, we adulterated two fish meals with pig PAPs containing cattle PAPs (**Mixes 70 to 73**). The amounts of PAPs added were calculated to have a final level of mammalian (pig + cattle) PAPs of 10 % and levels of cattle PAPs of 1 and 2 % respectively.

TABLE 16 :
ANALYSIS OF FISH MEALS ADULTERATED WITH PIG PAPS CONTAINING CATTLE PAPS AT FINAL LEVELS OF 1 OR 2 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in Feed
Mix 70 Fish meal (DQ/07/1089-14) + 9% pig PAPs (DQ/07/0134-09) + 1% U.S. Cattle PAPs (DQ/06/0959-17)	1: Negative 2: Negative
Mix 71 Fish meal (DQ/07/1089-14) + 8% pig PAPs (DQ/07/0134-09) + 2% U.S. Cattle PAPs (DQ/06/0959-17)	1: Negative 2: Negative
Mix 72 Fish meal (DQ/07/1089-14) + 9% pig PAPs (DQ/07/0134-09) + 1% Cattle PAPs (DQ/07/0134-04)	1: Positive 2: Positive
Mix 73 Fish meal (DQ/07/1089-14) + 8% pig PAPs (DQ/07/0134-09) + 2% Cattle PAPs (DQ/07/0134-04)	1: Positive 2: Positive

From these results, it appears that the incorporation of pig PAPs in fish meal could be a source of false negative results for the detection of cattle PAPs at a level of **1 % or 2 %** due to the masking effect of cattle PAPs by pig PAPs (Mixes 70 and 71 compared to Mix 69). Here also, the heat treatment process to which the cattle PAPs was submitted seems of crucial importance. Less heat treated cattle PAPs is more prone to the masking effect.

4.3.5. Analysis of Fishfeeds

Within the samples present in the CRL-AP sample bank, we tested 21 fishfeeds. Firstly, the samples were analysed by PCR to get additional information on their composition. Secondly, they were analysed with the 2 immunoassays.

TABLE 17 (1ST PART) :
ANALYSIS OF FISHFEED - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Samples	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Fishfeeds without any PCR detectable DNA from ruminants (cattle and sheep)		
DQ/08/0622-001	1: Invalid 2: Invalid	1: Positive 2: Positive
DQ/08/0622-002	1: Negative 2: Negative *	1: Negative 2: Negative
DQ/08/0622-003	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/08/0622-005	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/08/0622-006	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/08/0622-007	1: Negative * 2: Negative	1: Negative 2: Negative
DQ/08/0622-010	1: Negative * 2: Negative *	1: Negative 2: Negative

* Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.

TABLE 17 (2ND PART) :
ANALYSIS OF FISHFEED - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Samples	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
DQ/08/0622-011	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/08/0622-014	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/08/0622-015	1: Negative 2: Negative *	1: Negative 2: Negative
DQ/08/0622-016	1: Negative * 2: Negative	1: Negative 2: Negative
DQ/08/0622-017	1: Negative * 2: Negative	1: Negative 2: Negative
Fishfeeds with cattle DNA detected by PCR		
DQ/08/0622-004	1: Invalid 2: Invalid	1: Negative 2: Negative
DQ/08/0622-012	1: Invalid 2: Invalid	1: Negative 2: Negative
DQ/08/0622-013	1: Negative * 2: Negative *	1: Negative 2: Negative
Fishfeeds with other animal species DNA detected by PCR		
DQ/08/0622-008	1: Negative * 2: Negative *	1: Negative 2: Negative
DQ/08/0622-009	1: Negative 2: Negative	1: Negative 2: Negative
DQ/08/0622-018	1: Negative * 2: Negative *	1: Negative 2: Negative

* Coloration of the dipstick with a clearer test line. Based on a visual examination, the test has to be considered as negative even if the Accuscan Reader declares the sample as positive.

On this limited set of samples, only one sample gave a false positive result with the ***"ReVeal® for Ruminant in Feed"*** assay. Three samples analysed with the ***"ReVeal® for Ruminant in MBM"*** assay gave an invalid result (no control line). This is probably due to the low pH of the extract (between 3 and 4.5 whereas in most of the cases the pH is between 5.5 and 7).

Two fishfeeds (DQ/08/0622-006 and -009) were used to prepare new mixes. **Mixes 74 and 75** were prepared with a fishfeed (ref. DQ/08/0622-006) to which cattle PAPs (ref. DQ/06/0959-17 and DQ/07/0134-04) was respectively added at the level of 2 %. **Mixes 76 and 77** were prepared with the second fishfeed (DQ/08/0622-009) adulterated with the same cattle PAPs at the level of 2 %.

TABLE 18 :
ANALYSIS OF FISHFEED ADULTERATED WITH CATTLE PAPS AT A FINAL LEVEL OF 2 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN MBM AND THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in MBM	ReVeal® for Ruminant in Feed
Mix 74 (98% Fishfeed DQ/08/0622-006 + 2% Cattle PAPs (DQ/06/0959-17)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 75 (98% Fishfeed DQ/08/0622-006 + 2% Cattle PAPs (DQ/07/0134-04)	1: Positive 2: Positive	1: Positive 2: Positive
Mix 76 (98% Fishfeed DQ/08/0622-009 + 2% Cattle PAPs (DQ/06/0959-17)	1: Negative 2: Negative	1: Positive 2: Positive
Mix 77 (98% Fishfeed DQ/08/0622-009 + 2% Cattle PAPs (DQ/07/0134-04)	1: Positive 2: Positive	1: Positive 2: Positive

With the ***"ReVeal® for Ruminant in Feed"*** assay, the presence of cattle PAPs is detected at the level of 2 % whereas the ***"ReVeal® for Ruminant in MBM"*** is unable to detect the American cattle PAPs DQ/06/0959-17 in the 2 fishfeeds.

As with the fishmeals, we prepared also mixes with fishfeeds containing a final level of 10 % of terrestrial (cattle + pig) PAPs. In order to see if the potential masking effect of cattle PAPs by pig PAPs could lead to false negative results in the analysis of fishfeeds, we adulterated the two fishfeeds used for the mixes 74 to 77 with pig PAPs containing 1 and 2 % of the American cattle PAPs.

TABLE 19 :
ANALYSIS OF FISHFEEDS ADULTERATED WITH PIG PAPS CONTAINING CATTLE PAPS AT FINAL LEVELS OF 1 OR 2 % - RESULTS OBTAINED WITH THE REVEAL® FOR RUMINANT IN FEED ASSAYS

Mix composition	ReVeal® for Ruminant in Feed
Mix 78 Fishfeed (DQ/08/0622-006) + 9% pig PAPs (DQ/07/0134-09) + 1% U.S. Cattle PAPs (DQ/06/0959-17)	1: Negative 2: Negative
Mix 79 Fishfeed (DQ/08/0622-006) + 8% pig PAPs (DQ/07/0134-09) + 2% U.S. Cattle PAPs (DQ/06/0959-17)	1: Positive 2: Positive
Mix 80 Fishfeed (DQ/08/0622-009) + 9% pig PAPs (DQ/07/0134-09) + 1% U.S. Cattle PAPs (DQ/06/0959-17)	1: Negative 2: Negative
Mix 81 Fishfeed (DQ/08/0622-009) + 8% pig PAPs (DQ/07/0134-09) + 2% U.S. Cattle PAPs (DQ/06/0959-17)	1: Positive 2: Positive

From these results, a level of 2 % of cattle PAPs can be detected but at a level of 1 %, the test remains negative.

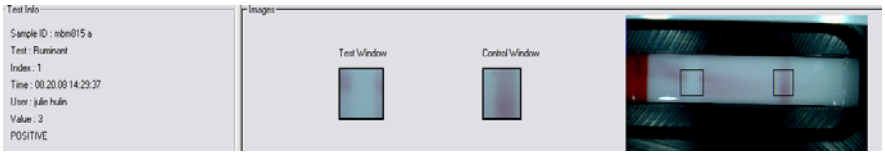
4.4. pH MEASUREMENTS TO CHECK POSSIBLE LINKS WITH FALSE OR INVALID TEST RESULTS

The pH measurements of the protein extracts did not show a large variability from sample to sample. The pH ranged between 5 - 7.5 and 6 - 7 for the extracts obtained with the ***"ReVeal® for Ruminant in MBM"*** and the ***"ReVeal® for Ruminant in Feed"*** kits respectively. No correlation could be found between false negative results and out of range pH values of the corresponding extract. On the contrary, there was a link with invalid results. A sample of vitamins for which the pH of the extract was at 5 (data not shown in tables) resulted in an invalid test (no control line). Furthermore, the three fish feeds that gave invalid results with the ***"ReVeal® for Ruminant in MBM"*** test (Table 17, first and second parts) also showed lower than normal pH values for their extracts.

5. CONCLUSIONS

In general, **the use of the *"ReVeal® for Ruminant in Feed"* assay instead of the *"ReVeal® for Ruminant in MBM"* one increased the sensitivity of the test for the qualitative detection of ruminant material in PAPs <u>but sometimes at the expense of the specificity of the analysis</u>.** Indeed false positive results are observed mainly with poultry PAPs. As the test should be considered as a screening step, this rate of false positive results is not really a concern but it means that a confirmatory method is needed. However it might be that for some production plants, this screening test will be of no use if all results are false positive ones.

A coloration of the strip is also frequently observed and the conclusion of the visual observations can be in contradiction with the one of the Accuscan Reader. The conflicting results are not systematically in the same direction : a very faint band can be declared as negative by the Accuscan Reader. To avoid any subjective interpretation we suggest that when the Accuscan delivers a negative result, this is kept even if visually a faint band could be visualised because this may even happen on blank samples. On the contrary, a coloured strip with a white test line gives a positive result with the reader (Figure 7). In this latter case it is suggested to give advantage to the visual result because the positive result given by the reader is an artefact. Fortunately, these problems mainly occur with the ***"ReVeal® for Ruminant in MBM"*** kit.



<u>FIGURE 7</u> : FALSE POSITIVE RESULT (COLOURED STRIP WITH A WHITE TEST LINE)

The use of the ***"ReVeal® for Ruminant in Feed"*** assay seems to be a solution to the false negative results obtained when pure ruminant PAPs are analysed with the ***"ReVeal® for Ruminant in MBM"*** test. This improvement is of great interest but a major drawback of the test remains the problem of false negative results with some porcine PAPs containing ruminant material. From the results we obtained, levels of contamination by bovine PAPs up to 1 % were not detected in some pig PAPs. More confusing, some cattle PAPs present at level as high as 30 % could not be detected in some pig PAPs. In this last case, the problem is due to a susceptibility of the test to the process conditions : **<u>samples submitted to heat treatment differing to much from the minimal European legal conditions (133°C, 3 atm, 20 minutes) are not detected under a level of contamination of 10 %</u>.** It is especially true for American and Australian bovine PAPs that were submitted to less stringent heat treatments

than what EU required. It may also happen on European PAPs submitted to harsh rendering conditions. In this latter case, the whole process is not well known but the PCR signals obtained with such PAPs give a clear evidence of a process having a huge degradation effect on organic molecules. **It seems that the higher sensitivity of the *"ReVeal® for Ruminant in Feed"* test does not solve this problem.** The CRL-AP is aware of the fact that most American or Australian bovine PAPs do not meet the rendering criteria to be accepted in the EU. However it was important to check with such materials if PAPs that would have been rendered inappropriately in Europe could still be detected easily or not with the Neogen screening test. The results of this study clearly show that PAPs that is less heat treated than legally required would be much more difficult to detect with the Neogen kits even when present at rather high levels in non-ruminant PAPs. Some tests of adulteration of fish meals with pig PAPs containing cattle PAPs gave also false negative results for cattle adulteration level of 1 or 2 %. **The main conclusion can be that the test would be largely process-dependent and therefore might induce some risk of false negative results.**

Inside the SAFEED-PAP project, the WP2 ("Improvement and validation of test kits") is working on the ReVeal for Ruminant test system. A big work was already done by the VLA (Veterinary Laboratories Agency, Luddington, UK) to improve the protein extraction step of the method and consequently the sensitivity of the assay. Preliminary tests gave interesting results in terms of sensitivity but they need to be confirmed on a larger set of samples. Another aspect is that the Neogen test is rapid (~ 45 minutes) and does not require skilled staff and expensive equipment allowing to use it as a rapid screening method. The improvement of the extraction step obtained by the VLA makes it much longer and its applicability as screening method has to be evaluated.

6. REFERENCES

AOAC Research Institute (2004). Certificate of Performance Tested℠ Status. Certificate n° 010405. http://www.aoac.org/testkits/010405-certificate.pdf

AOAC Research Institute (2005). Certificate of Performance Tested℠ Status. Certificate n° 070501. http://www.aoac.org/testkits/070501certificate.pdf

Boix A., von Holst C., Baeten V., Berben G. and Vancutsem J. (2004). *"Determination of Processed Animal Proteins (PAPs) including meat and bone meal (MBM) in feed – Part II : Prevalidation study for the detection of PAPs in feedby immunoassays."* Ref. N° SANCO/17 04 02/04/SI2.373351. 24 September 2004.

Boix A., Serano F., Bellorini S., von Holst C. (2006). *"Ruggedness Study of Immunoassays for Processed Animal Proteins Detection in Feed : Neogen ReVeal for Ruminant Feed Test System."* Ref. GE/R/FSQ/03/2006/09/04. European Commission. DG-JRC-IRMM, Geel, Belgium.

DNV Consulting (2006). *"Assessment of the risk potential of reintroduction of certain processed animal proteins into animal feeds."* Report to the European Fat Processors and Renderers Association. Report n° 22514037 rev 2, 4th August 2006.

EFPRA (2006). *"EFPRA Proposal for re-entry of certain PAP's for use in Feeds : A Discussion document."* Ref. EF/06/108. 09 October 2006.

Fumière O., Osmanaj I., Lafortune M.-G. & Berben G. (2004). *"Rapid detection of processed animal protein in feed with ReVeal® Ruminant strip test."* Rapid Methods Europe 2004, 25-26 March 2004, Noordwijk aan Zee, The Netherlands .

Fumière O., Dubois M., Baeten V., von Holst C. & Berben G. (2006). *"Effective PCR detection of animal species in higly processed animal byproducts and compound feeds."* Analytical and Bioanalytical Chemistry, 385, 1045-1054.

Lafortune M.-G. (2003). *"Contribution à la détection des espèces animales intervenant dans les produits agro-alimentaires."* Travail de fin d'études en vue de l'obtention du Diplôme d'Etudes Approfondies en Sciences et Technologie des Aliments. Faculté Universitaire des Sciences Agronomiques de Gembloux - Université Catholique de Louvain. Année académique 2002-2003.

Myers M. J., Yancy H. F., Farrell D. E., Washington J. D. & Frobish R. A. (2005). *"Evaluation of Two Commercial Lateral-Flow Test Kits for Detection of Animal Proteins in Animal Feed."* Journal of Food Protection, 68 (12), 2656-2664.

Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. Official Journal of the European Communities.31.05.01. L147.1–40.

Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. Official Journal of the European Communities 10.10.2002, L273, pp 1-95.

Vaessen J. (2007 a). *"ReVeal for Ruminant in Feed tested on ruminant spiked samples"*. Report ref. RAP-1001353. CCL Nutricontrol (Veghel, NL).

Vaessen J. (2007 b). *"Quick screening tests <ReVeal for Ruminant in Feed> and <ReVeal for Ruminant in MBM> tested on raw materials"*. Report ref. RAP-1001347. CCL Nutricontrol (Veghel, NL).

van den Hoven S. (2007). *"Performance of Neogens ReVeal for Ruminant in MBM"*. Report ref RAP-1001207. CCL Nutricontrol (Veghel, NL).

Woodgate S.L. (2007 a). *"EFPRA Proposal for the approval of Non-ruminant Processed Animal Proteins to be used in Feeds for Aquatic Species (Aqua-feeds)"*. Ref. EF/07/85. October 18th 2007.

Woodgate S.L. (2007 b). *"Feed chain: specificity and challenges"*. FEED SAFETY International Conference 2007 : Methods and Challenges. 27-28 November 2007. http://safeedpap.feedsafety.org/fs2007/lectures.php.

ADDENDUM

PRELIMINARY TESTS WITH THE « *MELISA-TEK™* » RUMINANT KIT FROM ELISA TECHNOLOGIES, INC., GAINESVILLE, FL, USA

1. INTRODUCTION

The MELISA-TEK™ Ruminant kit (ELISA Technologies Inc., Gainesville, FL, USA) is intended to detect ruminant muscle tissue in extracts made from cooked meat and feed products such as meat meals and meat and bone meals. The assay is a sandwich ELISA based on species specific recognition of troponin I by specific monoclonal antibodies.

Briefly, the test can be described like this : one troponin I specific monoclonal antibody is immobilized to the wells of the test strips, which captures troponin I present in the samples or controls. After a wash step, a second troponin I specific monoclonal antibody, which has been biotinylated, is allowed to bind to the troponin I present in the well. After a second wash step, a streptavidin-horseradish peroxidase (SA-HRP) conjugate is added which binds to the biotinylated secondary antibody, and any unbound SA-HRP is washed away. The tetramethylbenzidin (TMB) substrate is added, which reacts with the HRP of the conjugate, causing a colour change in proportion to the level of troponin I originally bound to the well. Finally, a stop solution is added after a specific time and colour development is evaluated using an ELISA plate reader (reading at the wavelength of 450 nm).

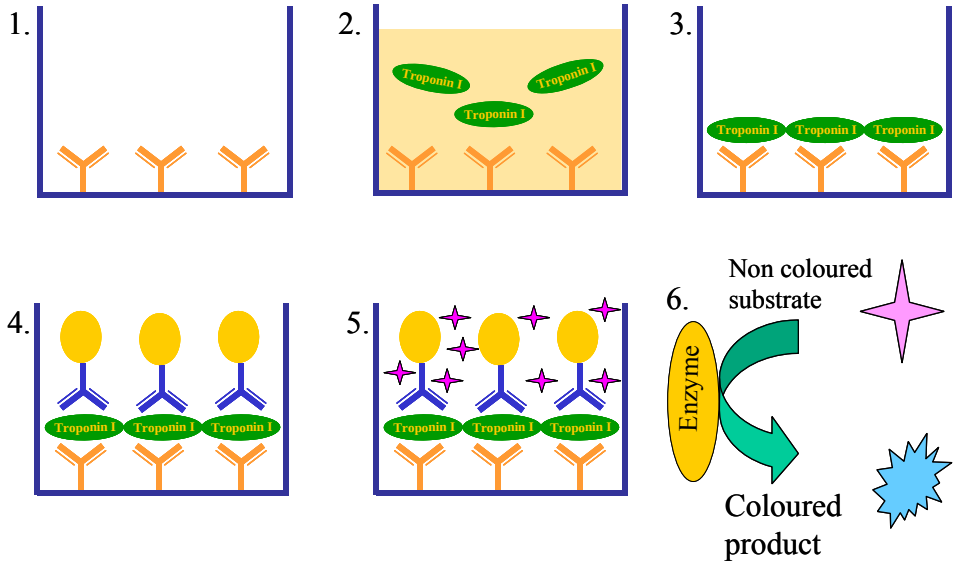


FIGURE 1 : GENERAL SCHEME OF A SANDWICH ELISA

Based on results communicated by the CCL [1], it seems that the MELISA-TEK kit could be a good alternative to the Neogen test for the detection of ruminant PAPs.

According to the manual provided by the manufacturer [2], the MELISA-TEK SPECIES kits are able to identify the presence of thermo-stable muscle tissue protein in meat and bone meals and animal feed samples containing muscle tissue at levels of approximately 0.05% or greater. In house testing by the manufacturer indicates the following detection limits:

1. Muscle tissue : lean muscle tissue prepared at up to 138 °C and 4 bars pressure during 20 minutes in a closed container is detected at a 1:2000 dilution of an 1:10 extract equivalent to a concentration of 0.005 % lean muscle tissue in a sample.

2. Meat and bone meal : a meat and bone meal (provided by IRMM) processed at 133 °C and 3 bars during 20 minutes composed of 50 % beef/50 % pork is detected at a 1:100 dilution of an extract equivalent to a concentration of 0.5 % in weight of meat and bone meal in a sample.

3. Animal feed : species thermo-stable muscle tissue protein antigens are detected in animal feeds containing 5 % of a meat and bone meal processed at 133 °C and 3 bars during 20 minutes composed of 50 % beef/50 % pork equivalent to a concentration of 2.5 % in weight of meat and bone meal in a feed sample.

Concerning the specificity of the test, numerous organic and inorganic compounds commonly present in animal feeds have been tested and do not interfere with the test [2].

The purpose of the limited number of tests performed by the CRL-AP within this study is only to check if the MELISA-TEK kit is able to detect the presence of ruminant material in some of the main samples giving false negative results with the Neogen dipstick test.

[1] CCL Nutricontrol (2008). MELISA-TEK RUMINANT kit tested. RAP-1001872.
[2] ELISA Technologies Inc. MELISA-TEK™ SPECIATION KITS for MEAT & BONE MEALS and ANIMAL FEEDS. Instructions for use. Revision 70308-V1.

2. DESIGN OF ANALYSIS AND RESULTS INTREPRETATION

The analyses were made on a limited number of samples just to check if the MELISA-TEK kit could be an interesting alternative to the Neogen test.

The MELISA-TEK kit provides a 96-microwell unit that may be divided into a variety of strip formats depending on the number of samples to be analysed. For each set of analyses, different controls (Blank, LPC, HPC, Neg) have to be included in each plate format (Figure 2).

STRIP	1	2	3	4	5	6	7	8	9	10	11	12
A	*Blank*	*Blank*	*Blank*									
B	*LPC*	*LPC*	*LPC*									
C	*HPC*	*HPC*	*HPC*									
D	*Neg*	*Neg*	*Neg*									
E	*1*	*1*	*1*									
F	*2*	*2*	*2*									
G	*3*	*3*	*3*									
H	*4*	*4*	*4*									
SPECIES	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*	*Rum*

Legend :
Blank = Extraction Solution Only
LPC = Low Positive Control (0.05 %)
HPC = High Positive Control (1 %)
Neg = Negative Control (alternate species control)
= Sample number
Rum = Ruminant

FIGURE 2 : EXAMPLE OF A SAMPLE PLATE PLAN

For screening, the supplier recommends that each control and sample extract were tested in duplicate. For confirmatory testing, triplicate or quadruplicate are recommended. In our case, samples and controls were tested in triplicate.

The assay is considered **VALID** if :

a) The blank-subtracted OD of the 1 % HPC is greater than 1.000 ;
b) The blank-subtracted OD of the 0.05 % LPC is greater than 0.100 ;
c) The blank-subtracted OD of the NEG is less 0.100 ;
d) The standard deviation of the 0.05 % LPC replicates is no more than 0.100

If these conditions are not met, the test is **INVALID** and should be repeated.

If the assay is valid, the samples may be classified as positive or negative. Test samples are classified as **POSITIVE** if the blank-subtracted average OD is greater than 0.100.

CCL proposes other rules to classify the samples (minutes meeting CRL-CCL – 19/09/2008) :

Test samples are classified as **POSITIVE** if its blank-subtracted average OD is greater than the blank-subtracted average OD of all negative samples + 3 x standard deviation. This way of doing is certainly an acceptable criterion.

3. RESULTS

A limited number of samples was analysed in order to check if the MELISA-TEK kit is able to solve the false positive results due to i) the use of ruminant material not properly rendered according to E.U. requirements and ii) the masking effect due to some pig PAPs.

TABLE 1 :
LIST OF THE MIXES TESTED WITH THE MELISA-TEK ASSAY (CORRESPONDANCE WITH THE MIXES TESTED WITH THE NEOGEN TEST AND COMPOSITION OF THE MIXES)

Sample	Mix n° (Neogen study)	Composition
1	Mix 1	99 % Pig PAPs 141 °C + 1 % Cattle PAPs (DQ/06/0954-17)
2	NT *	99 % Pig PAPs 141 °C + 1 % Cattle PAPs (DQ/02/0862-02)
3	NT *	99 % Pig PAPs 141 °C + 1 % Cattle PAPs (DQ/02/1032)
4	Mix 31	99 % Pig PAPs 141 °C + 1 % Cattle PAPs 137 °C
5	Mix 33	99 % Pig PAPs 141 °C + 1 % Cattle PAPs 133 °C
6	Mix 2	98 % Pig PAPs 141 °C + 2 % Cattle PAPs (DQ/06/0954-17)
7	NT *	98 % Pig PAPs 141 °C + 2 % Cattle PAPs (DQ/02/0862-02)
8	NT *	98 % Pig PAPs 141 °C + 2 % Cattle PAPs (DQ/02/1032)
9	Mix 32	98 % Pig PAPs 141 °C + 2 % Cattle PAPs 137 °C
10	Mix 70	90 % Fishmeal + 8 % Pig PAPs 141 °C + 2 % Cattle PAPs

* NT = Not tested with the Neogen dipstick test but a similar mix with a higher Cattle PAPs content (Table 12 - page 17) gave a false negative result.

The controls gave the following results :

TABLE 2 :
ABSORBANCES AT 450 NM, STATISTICS WITH THE CONTROLS AND CONCLUSIONS ABOUT THE VALIDITY OF THE ASSAY

Controls	Absorbance @ 450 nm				STD DEV	Average OD minus blank OD	Assay validity	
	OD 1	OD 2	OD 3	Mean			Control OD	Pass/Fail
Extraction solution (Blank)	0.070 0.063 0.073	0.089 0.076 0.062	0.077 0.065 0.053	0.070	0.010	0.000		
Negative	0.074	0.067	0.065	0.069	0.005	-0.001	≤ 0.100	*Pass*
LPC	0.483	0.463	0.444	0.463	0.020	0.393	≥ 0.100	*Pass*
HPC	3.080	3.113	3.096	3.096	0.017	3.026	≥ 1.000	*Pass*

According to these figures, the test is valid and allows the classification of the samples.

TABLE 3 :
ABSORBANCES AT 450 NM, STATISTICS WITH THE SAMPLES AND CONCLUSIONS ABOUT THE CLASSIFICATION OF THE SAMPLES ACCORDING TO THE DECISION CRITERION OF ELISA TECHNOLOGIES INC.

Samples	Absorbance @ 450 nm				Blank OD	Average OD minus blank OD	Sample result OD ≤ 0.100 = Negative OD > 0.100 = Positive
	OD 1	OD 2	OD 3	Mean			
1	0.093	0.087	0.084	0.088	0.070	0.018	*Negative*
2	0.161	0.174	0.165	0.167		0.097	*Negative*
3	0.095	0.099	0.090	0.095		0.025	*Negative*
4	0.098	0.102	0.096	0.099		0.029	*Negative*
5	0.160	0.151	0.165	0.159		0.089	*Negative*
6	0.101	0.100	0.095	0.099		0.029	*Negative*
7	0.295	0.290	0.284	0.290		0.220	*Positive*
8	0.128	0.130	0.131	0.130		0.060	*Negative*
9	0.125	0.128	0.120	0.124		0.055	*Negative*
10	0.111	0.101	0.096	0.103		0.033	*Negative*

From this limited sample set, only one mix (mix 7) gave a positive result. It contains 98 % Pig PAPs 141 °C + 2 % of an American Cattle PAPs. The other samples containing 2 or 1 % of Cattle PAPs gave all false negative results.

Table 3 shows the conclusions obtained with the decision criterion proposed by the CCL.

TABLE 4 :
ABSORBANCES AT 450 NM, STATISTICS WITH THE SAMPLES AND CONCLUSIONS ABOUT THE CLASSIFICATION OF THE SAMPLES ACCORDING TO THE DECISION CRITERION OF CCL NUTRICONTROL.

Samples	Absorbance @ 450 nm				STD DEV	Sample result
	OD 1	OD 2	OD 3	Mean		
Extraction solution (Blank)				0.070	0.010	Mean OD ≤ 0.100 = Negative Mean OD > 0.100 = Positive
1	0.093	0.087	0.084	0.088		*Negative*
2	0.161	0.174	0.165	0.167		*Positive*
3	0.095	0.099	0.090	0.095		*Negative*
4	0.098	0.102	0.096	0.099		*Negative*
5	0.160	0.151	0.165	0.159		*Positive*
6	0.101	0.100	0.095	0.099		*Negative*
7	0.295	0.290	0.284	0.290		*Positive*
8	0.128	0.130	0.131	0.130		*Positive*
9	0.125	0.128	0.120	0.124		*Positive*
10	0.111	0.101	0.096	0.103		*Positive*

With this new decision criterion, 6 samples are classified as positive and 2 samples with negative results (mixes 3 and 4) are very close to the decision threshold ($OD_{450\ nm}$ > 0.100). Among the false negative results, only mix 4 contains **1 %** in weight of a European Cattle PAPs heat treated at **137 °C**. It is very close to the decision threshold ($OD_{450\ nm}$ > 0.100). The same Pig PAPs containing **2 %** in weight of the same Cattle PAPs is detected as positive. These new rules of decision could be an improvement for the test used as a screening method.

4. CONCLUSIONS

The preliminary tests performed aimed to check whether the MELISA-TEK test could be a good alternative to the Neogen assay for the detection of ruminant PAPs in PAPs. For that reason, they were mainly focussed on samples giving false negative results with the Neogen kit.

The first results obtained show that there is almost no improvement of the results when the Elisa Technologies Inc. decision criteria are used. **Nevertheless, the criteria proposed by the CCL allows a much better classification of the samples.**

Concerning economical and practical aspects of the MELISA-TEK kit, some considerations can be detailed :

1. The price of the kit is more or less comparable with the one of the Neogen kit : 369 € / plate of 96 wells *vs.* 351 € / 25 dipsticks. These figures must be balanced by the fact that replicates (2 to 4) are recommended by the MELISA-TEK supplier and controls are needed for each set of analysis. With the Neogen kit, the CRL-AP proposes as a compromise to perform each sample analysis in duplicate.

2. The time needed for a set of analysis is much more longer with the MELISA-TEK kit than with the Neogen kit : > ½ day *vs.* < ½ day respectively. The MELISA-TEK kit becomes interesting only with a wide set of samples (> 20-25 samples) to analyse.

3. The MELISA-TEK kit needs a more skilled staff and specific laboratory equipment (micro-well plate reader, micropipettes,…) to perform the analysis and its protocol is much more tedious whereas the Neogen kit does not need a heavy training and can be used without any highly specialised laboratory equipment except the Accuscan Reader which is itself optional but nevertheless recommended for a less subjective interpretation of results in case of low presence.