Chapter 9
DNA based approaches for the species-specific identification of animal material

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DNA BASED APPROACHES FOR THE SPECIES-SPECIFIC IDENTIFICATION OF ANIMAL MATERIAL

ABBREVIATIONS

Bp	Base pairs
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
CRA-W	Walloon Agricultural Research Centre
dsDNA	Double stranded DNA
EU	European Union
EC	European Commission
JRC-IRMM	Joint Research Centre – Institute for Reference Materials and Measurements
MAT	The microscopic analysis test
MBM	Meat and Bone Meal
MtDNA	Mitochondrial DNA
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PAPs	Processed animal proteins
RT-PCR	Real time polymerase chain reaction
SINE's	Short interspersed nuclear elements
ssDNA	Single stranded DNA
TCE	Tetrachloroethylene
TSE	Transmissible Spongiform Encephalopathy
vCJD	Variant Creutzfeldt-Jakob Disease
VLA	Veterinary Laboratory Agency

SUMMARY

The detection of processed animal proteins using the PCR is largely investigated. This indirect method targeting the DNA is indeed the most promising analytical approach to complement microscopy with information on the protein origin at a species level. Thanks to the development of the real-time PCR technology, it is possible to design short size targets able to amplify efficiently highly degraded DNA templates such as in the case of MBM. In this chapter the advantages and drawbacks of the PCR will be reminded and the last improvements of the PCR analytical process of feed samples methods developed during the SAFEED-PAP project will be discussed.

Keywords: PCR, DNA, test portion.

9.1. INTRODUCTION

Since the outbreak of the BSE and the naming of the meat and bone meal (MBM) as the main vector of the spread of the disease several European and national projects have been undertaken in order to develop suitable methods to detect the presence of mammalian MBM in compound feeds. The official term for animal by-products of the lowest risk category (category 3) is processed animal proteins (PAPs) which are produced mainly in the form of ground processed (rendered) slaughter by-products originating essentially from ruminant, pig, poultry or fish. Lack of specific analytical methods especially for the heavy heat treatments applied to mammalian meat and bone meal rendering methods have led to a total ban of animal by-products in feed (with some exceptions).

In order to improve the impact of the total ban, the European Commission requires the development and validation of new tests which allow differentiation of processed animal proteins on a species-specific basis. This would permit the European Commission to consider the partial lifting of the ban by reintroducing certain species specific proteins to the animal feed without any modification of the existing prohibition on intra-species recycling assuring a high level of food safety.

As this review will show, PCR might be of great help to that purpose even if there are some limitations one has to be aware of. Indeed, DNA as an analyte even when damaged by the rendering process will generally remain in the product (Chiappini *et al*., 2005; Pascoal *et al*., 2005). Generally, mitochondrial DNA (mtDNA) based PCR methods have given good results in analysis of samples submitted to temperature and pressure treatments, in which DNA has been partly degraded (Tartaglia *et al*., 1998; Wang *et al*., 2000; Bellagamba *et al*., 2001; Myers *et al*., 2001; Bottero *et al*., 2003). PCR primers designed on the basis of sequences of short interspersed repetitive elements have also been successfully used to detect low contamination levels of feed by animal material (Tajima *et al*., 2002; Aarts *et al*., 2006). With the emergence of real-time PCR technology, PCR methods for mitochondrial encoded targets (Lahiff *et al*., 2001 ; Fumière *et al*., 2006) or those in nuclear sequences (Brodmann and Moor, 2003) based on the use of Taqman probes, have been reported for the detection of bovine material in MBM and feedstuffs containing animal MBM (Castelló *et al*., 2004).

The PCR approach being a DNA-based technique, detection will only be possible as long as DNA is still

available (or at least DNA in pieces with a sufficient amount in the size range between 60 and 100bp) in the PAPs, efficiently extractable and PCR amplifiable with specific targets (Hird *et al.*, 2006). Special treatments with acids or bases, if used during rendering, can also have a great damaging effect on DNA although it seems that the DNA in bone particles is better protected (Götherström *et al.*, 2002; Buckley *et al.*, 2008) as in fossils where DNA has been kept for very long periods. Nevertheless, this limitation can be partially or totally bypassed when using multicopy and short sized targets to increase the sensitivity of the test. Another limit of the PCR approach is the fact that presence of animal DNA (belonging to a species or a group of species) does not necessarily come from PAPs, it may come from milk, blood, fat or egg products. The practical impact of this limitation is not well known as some of these products are rather expensive so that they are not that widely used in fee. Nevertheless it may be a cause of false positive results. It should be stressed however that presently blood-derivatives are also forbidden in feed for ruminants. For blood from non ruminants, its use is limited to non-ruminant feed. For fat too, the problem is probably limited because with high quality fat no DNA will be detectable, while with lesser quality fats containing impurities, DNA may still be found but this type of fat may also represent a greater risk for transmission of TSEs.

9.2. CRITICAL PCR PROTOCOL POINTS STUDIED WITHIN THE SAFEED-PAP PROJECT

During the SAFEED-PAP project different critical points for the fitness of the PCR methods were investigated namely the representative character of a test portion in connection with DNA extraction protocols, the transferability of a PCR qualitative test having the potential to be distributed as a commercial kit as well as the analysis of single PAP particles free of environmental DNA. This last topic is treated in the chapter 13 (Combination methods for PAP detection and species determination of animal particles).

9.2.1. Representation of a test portion in connection with the DNA extraction method

A supposed restrictive drawback of the PCR is the limited test portion (100 – 200 mg) often used for the DNA extraction. Such a limited test portion size might impede a good representation of the whole laboratory sample and therefore lead to an increase of the limit of detection. The DNA extraction methods daily used by VLA and CRA-W starting with quite different sizes of test portions (respectively 40 g and 100 mg) were compared in order to see if they have a strong influence on the limit of detection of animal proteins in feed with DNA-based methods.

9.2.1.1. Material and methods

Description of test materials

CRA-W samples set

Two sets (one for each lab) of spiked samples were prepared with a feed matrix previously analysed by classical microscopy and PCR to ensure that it contained no detectable amounts of meat and bone meal (MBM). Material used for spiking was pure cattle MBM obtained from a pilot plant and treated at a temperature of 133 °C and 3 bars for 20 minutes as required by European legislation.

The prepared spiked samples consisted of two sets of 10 samples containing decreasing levels of a cattle MBM: 0.2 %, 0.1 %, 0.075 %, 0.050 %, 0.025 %, 0.0125 %, 0.0075 %, 0.0033 % and an unspiked sample at 0 %. In each set the different MBM levels are present only once except the 0.1 % level that is duplicated.

All samples were prepared individually by adding the exact amount of MBM to each sample considering a final weight of 40 g to be fit for the extraction protocol of the VLA. The lowest amount added consisted of 1.3 mg of MBM (sample at the 0.0033 % level).

VLA samples set

Two sets of 10 samples were prepared individually by contaminating 38.8 grams of negative feed matrix (made of equal amount of sun flower, maize, rape seed, wheat and soya) with 0.2 grams (0.5%) of species-specific meat and bone meal from various species (bovine, ovine, porcine, avian and fish) rendered according to the EU legislation.

The composition of the samples is given in table 9.1.

DNA extraction

Because contaminated particles may not be uniformly dispersed throughout a feed batch it is vital that appropriate sampling techniques are employed in order to generate an accurate and reproducible result. Each of the laboratories used its own extraction protocol.

The procedure applied by CRA-W consisted of a first step in which samples were ground on a ZM200 mill (Retsch GmbH & Co., Haan, Germany) to obtain a powder of particles with a diameter < 0.5 mm before extraction. After this step DNA was extracted and purified in duplicate from a 100 mg test portion with the commercial kit "Wizard® Magnetic DNA Purification System for Food" (Promega Corporation, Madison, WI, USA) according to supplier's instructions and using the King Fisher Magnetic Particle Processor (Thermo Labsystems, Helsinki, Finland) as semi-automatic device for performing these extractions. Final DNA extract was

recovered in 300 µl (solution corresponding to the undiluted extract).

VLA developed a method for extracting DNA from the feed sample starting from a larger test sample (40 g) in order to ensure the detection of target DNA in samples, and using a Chelex resin DNA extraction/purification protocol. With this method, the test portion is soaked in a phosphate buffer to release material from the sampled feed. The soaked sample is pre-processed by a heat-treatment in order to release the DNA into the buffer. A sub-aliquote of buffer is treated with Chelex, vortexed for 20 seconds and then centrifuged for 10 minutes. The liquid is then removed and diluted 1:10 v/v for testing.

Real-time PCR analysis

For the real-time PCR analysis each laboratory used its own primers and probes and its own protocols.

For the method developed by CRA-W, the DNA extract of each test portion was submitted to PCR on 5 µl of undiluted extract as well as on a tenfold dilution to check for possible inhibition. Real-Time PCR was performed with an ABI5700 thermocycler (Applied Biosystems, Foster City, CA, USA) in a total volume of 35 µl containing 5 µl of template DNA (undiluted or 10 fold diluted Promega extract to check for inhibition), 17.5 µl of qPCR™ Mastermix (Eurogentec, Belgium), 0.75 µl of each primer at 5 µM (Eurogentec), 2.5 µl of the appropriate double dye probe at 5 µM (Eurogentec) labelled with fluorescent reporter dye 6-carboxyfluorescein (FAM) and 8.5 µl of PCR-grade water (ICN Biomedicals, Belgium). Some characteristics linked to the target and the reagents of the CRA-W PCR test are given in table 9.2.

For the method developed by VLA, real-time multiplex PCR was performed and detected on a 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl containing 12.45 µl of TaqMan mastermix (Applied Biosystems), 0.07 µl of forward primer and 0.08 µl of reverse primer at 0.3 µM and 0.13 µl of probe labelled with fluorescent reporter dye 6-carboxyfluorescein (FAM) at 0.1 µM. In addition, an internal control (IC) is used for each test well by adding 1 µl of IC template at a 15000 fold dilution, 0.01 µl of IC forward primer, 0.04 µl of IC reverse primer both at 0.03 µM and 0.19 µl of IC probe VIC labelled 0.15 µM. This involves the amplification of a region of the ampicillin resistance gene commonly found in commercial nucleic acid vectors but not naturally found in animal or plant genomes. It is a non-competitive exogenous control and it is included in the assays to detect false negative results that may arise as a result of inhibitory factors present in the samples. The internal control template, primers and probes are added to the TaqMan master mix to allow multiplex detection. The primers were used at a limiting concentration to prevent IC product using the PCR reagents to the detriment of the mtDNA target amplification efficiency. Each assay is run with four non-template controls, six target species positive controls (0.2% 133°C PAP in a blank feed), 2 lots of each non-target species and 2 negative feed controls. Cut-off values are generated on an assay by assay basis using positive control data.

Both methods have been in-house validated and are accredited under the ISO 17025:2005 quality standard in their respective laboratory.

Handling of samples in each laboratory for independent assessment of the analytical steps

Each laboratory is using its own extraction protocol and PCR protocol. Therefore the sample sets are made in double allowing each laboratory to achieve its own extraction protocol on the samples. The samples are sent in blind to the other laboratory. Moreover the extracts obtained are shared (but sent in blind) in order to allow each laboratory to perform its own PCR protocol on the several DNA extracts. By this strategy the influence of the two analytical steps, i.e. extraction and PCR on the final results can be assessed. A summary of main characteristics of the analytical steps used in each laboratory is given in table 9.3.

9.2.1.2. Results and discussion

Results obtained with the VLA samples set

Table 9.4 presents the results obtained at CRA-W with the sample set produced by VLA. The two samples containing bovine material were detected whatever the extraction method. No false positive result nor false negative one was observed. Moreover, the simultaneous presence of another MBM (a porcine MBM) does not seem to affect strongly the detection of the bovine material. Nevertheless, the C_t values of the DNAs extracted by the VLA were very close to each other whereas on DNA extracted by CRA-W, the Ct values extended over a range of 2 cycles.

From the results on this sample set, it can be concluded that with the two extraction methods considered there was no PCR-inhibition problem. However, this is strongly dependent on the kind of matrix analysed. The C_t values obtained with the extraction method of the VLA are slightly sooner than the ones with the CRA-W extraction method but the differences observed could only be due to different amounts of DNA present in the reaction.

To complete these results, CRA-W also extracted the DNAs of the same sample set using a classical phenol/chloroform extraction procedure as described in Fumière *et al.* (2006). This protocol was developed with a duplicate test portion of 200 mg per sample. The extracted DNA is suspended in a final volume of 600 µl (undiluted extract). Table 9.5 presents the results obtained with the phenol/chloroform extracts.

The two samples containing bovine materials were detected. But the DNAs obtained with this extraction method showed a strong PCR inhibition needing a 100 fold dilution of the extracts to allow an amplification of the target. With such a dilution of the extracts, the C_t values observed were comparable to those recorded with the undiluted extracts of the favourite methods of VLA and CRA-W.

Table 9.6 gives results similar to those of table 9.4 but with the PCR test of VLA performed at VLA on the VLA sample set extracted either by the VLA method or by the CRA-W method. Here too, all results for bovine are correct. Moreover VLA also checked the presence of avian, ovine and porcine targets and all results were correct (data not shown). CRA-W afterwards also looked to the porcine, ovine, poultry and fish targets and results were also fine (data not shown).

Results obtained with the CRA-W sample set

CRA-W produced a set of samples with decreasing content of bovine material in order to assess, whether the extraction protocols and especially the test portion size could have an influence on the limit of detection. Table 9.7 presents the results obtained with the CRA-W bovine PCR test on DNAs extracted from this second sample set.

On this set of samples, the CRA-W bovine PCR assay can detect bovine PAP contamination levels lower than 0.1 % (w/w) whatever the DNA extraction method (from VLA or CRA-W). A level of 0.025 % is detected with the undiluted extract of both extraction methods. The level of 0.0125 % of bovine MBM is even detected with the DNA extracted according the CRA-W protocol. This result must be considered cautiously as the test is not validated at this level of contamination and the C_t values are close to the limit of detection even if the two independent test portions were detected as positive.

On the other hand, the VLA bovine PCR test was unable to detect any of the positive samples of the CRA-W sample set whatever the kind of extraction (data not shown). This is linked to a lesser sensitivity of this test which is validated to detect contamination levels by bovine material at 0.2 %.

9.2.2. Development of a method for the determination of a cut-off value using plasmids

One of the main problems with qualitative detection by real time PCR is the objective definition of a cut-off value determining the limit between a positive result and a negative one. In fact, the Ct value reflects the number of target copies present in the reaction but it is a relative parameter depending on the PCR platform (combination of a mastermix and a thermocycler and how the operator sets the fluorescence threshold). It is the same for the cut-off value that can be defined as the upper limit of the confidence interval of the Ct value corresponding to one copy of the target and has to be determined for each PCR platform.

To assure the accurate transferability of the test and make a kit usable by every testing labs, copy-number based calibration curves were developed with the help of plasmids to link the cut-off value to the number of targets in the reaction. A statistical approach was also designed to calculate a cut-off value in accordance with its definition. The cut-off value is determined in such a way that there is no more than 5 % of chance that later signals are due to the presence of the target. Later signals are considered as insignificant but there is in fact still up to 5% of chance that it is a late amplification signal linked to the DNA fragment that was targeted.

Based on a large dataset made of the data from 23 independent calibrations performed in-house on a Light Cycler LC480 (Roche,….), the protocol for the determination of the cut-off value of a PCR platform was refined to solve the following practical questions linked to the use of a kit : What is the minimum number of calibrations needed to estimate accurately the cut-off value of a test performed on a specific PCR platform? Starting from calibrations using 28 wells (7 levels of copy number tested with 4 replicates), is it possible to reduce the number of calibration points needed to make a calibration without important accuracy loss? Is it possible to reduce the number of calibration points needed to make a calibration without important accuracy loss?

Statistically, it was calculated that calibration using only 3 calibration points (640, 160 and 40 copies of plasmid/5µl) tested in triplicate can be used with the same accuracy reducing from 28 to 9 the number of wells to be used for the calibration. The minimum number of calibrations to perform to obtain a robust cut-off is still to determine but 12 calibrations seem to be a target figure obtained from the data collected at CRA-W. The final protocol developed to determine the cut-off value of a PCR platform was first applied in-house on two thermocyclers from Applied Biosystems: one ABI 7000 and one ABI 7500 before a successful validation study involving 22 PCR platforms from 4 major companies located in 19 laboratories from Europe, Japan and Australia. The cut-off determined on the 22 PCR platforms were distributed on a wide range of Ct values (from 37.73 to 43.68 cycles) showing the usefulness of the transfer protocol including the determination of the cut-off of a PCR platform (Fumière *et al*., 2010a). The transfer protocol will be extensively described by Fumière *et al.* (article in preparation). Following this first interlaboratory study, a validation study of the CRA-W PCR method of cattle DNA detection gathering 17 laboratories from EU and Japan was organized by the JRC-IRMM in collaboration with CRA-W and FERA. This last study differed from the first one by some aspects: 1. It aimed to validate a PCR method able to detect 0.1% (w/W) cattle PAP in feed; 2. Master mixes from

three companies were used; 3. The cut-off calculated was visible to the participants; 4. The participants had to provide qualitative results and not only raw data. From the results obtained, it was concluded that the PCR method tested is valid for the detection of bovine MBM in feed at the level of 0.1 % but a particular attention must be paid to the master mix used as some contain BSA (Bovine Serum Albumin) in their buffer to stabilize the *Taq* Polymerase (Concannon *et al.*, 2011). The amount of BSA included can strongly vary from one master mix to another one so that it is impossible to use the ones with the higher concentrations of BSA for this specific test targeting bovine DNA. Indeed, even the non template controls would give systematically false positive results. The complete results will be published by Boix *et al.* (paper in preparation).

9.3. CONCLUSIONS

9.3.1. Size of the test portion to consider

To test the hypothesis that increasing the size of the test portion would improve test performance, raw samples were exchanged by two laboratories and DNAs were extracted according to two completely different protocols. The DNAs obtained from the raw samples by the two extraction methods were also exchanged in order to allow each laboratory to perform its own PCR method. Using this experimental design, the results allow an evaluation of the extraction step efficiency independently of the sensitivity of the PCR analysis and to consider the analytical steps separately similar to the modular approach in GMO-detection (Holst-Jensen & Berdal, 2004). The overall conclusion based on the analysis of all the sets of samples is that in general both extraction protocols have close efficiencies. However, with very heterogeneous samples, the commercially available DNA extraction kit used by CRA-W allows to obtain DNAs of higher quality when compared to VLA method. Nevertheless, it must be mentioned that the VLA extraction protocol allows to handle quickly a large number of samples whereas the capacity of the CRA-W protocol is more limited for high throughput analysis purposes. Indeed it involves a time-consuming grinding step which nevertheless allows to obtain highly representative, homogenous and reproducible test portions.

9.3.2. Development of a method for the determination of a cut-off value using plasmids

A copy-number based calibration curves in combination with a statistical approach to calculate the cut-off value of a PCR platform was developed and validated. Defined as the upper limit of the confidence interval of the Ct value corresponding to one copy of the target, the cut-off value is determined in such a way that there is no more than 5 % of chance that later signals are due to the presence of the target. Later signals are considered as insignificant but there is in fact still up to 5% of chance that it is a late amplification signal linked to the DNA fragment that was targeted. This transfer protocol applicable to any PCR qualitative test was used to validate successfully the CRA-W PCR method for the detection of cattle MBM in feed.

Some problems are however still pending for a complete transferability of the method: the production of the plasmids used as calibrants must be fixed. The accurate measurement of the number of copy using digital PCR is a prerequisite to obtain reproducible batches (Fumière *et al.*, 2010b). With such "reference materials" an efficient follow-up of the performances of routine results with control chart will be possible.

The last interlaboratory study also pointed out the importance of the master mix used and its cautious checking especially for the detection of bovine/ruminant material. Some master mixes contain BSA (Bovine Serum Albumin) in their buffer to stabilize the *Taq* Polymerase (Concannon *et al.*, 2011). The amount of BSA included can strongly vary so that it is impossible to use the master mixes with the higher concentrations of BSA for that analysis as it would imply systematically false positive results.

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Table 9.1. Composition of the sample set prepared by VLA

Sample No.	Composition
1	Negative Feed Matrix
2	Bovine 0.5%
3	Ovine 0.5%
4	Porcine 0.5%
5	Avian 0.5%
6	Fish 0.5%
7	Bovine 0.5%+Porcine 0.5%
8	Ovine 0.5%+ Avian 0.5%
9	Avian 0.5%+ Fish 0.5%
10	Negative Feed Matrix

Table 9.2. Main characteristics of target and oligonucleotides used at CRA-W (Fumière *et al*., 2006)

Species targeted	Amplicon characteristics	Oligonucleotide	Length (in bp)	Melting temperature T_m (in °C)
Cattle	Length : 68 bp	Direct primer ATP-UN/B1	17	35
	T_m : 77 °C	Reverse primer ATP-UN/B2	16	38
		Probe ATP-UN/B	24	53

Table 9.3. Summary of the protocols used by CRA-W and VLA

		CRA-W	VLA
DNA Extraction/purification protocol	Test portion size	100 mg x 2	40 g
	Method used	Magnetic beads	Heat treated and Chelexed
Amplification protocol	DNA target	Mitochondrial DNA	Mitochondrial DNA
	Species detected/amplicon size	Cattle/68 bp	Bovine/108 bp
	Cut-off limit [a]	40 cycles	About 35 cycles

[a] Limit to distinguish between positive and negative samples.

Table 9.4. PCR results obtained with the CRA-W bovine PCR test on DNAs produced with the VLA and the CRA-W extraction methods from the VLA sample set

Sample	Animal protein content	Ct values obtained with VLA extracts*		Conclusion	Ct values obtained with CRA-W extracts*				Conclusion
		Undiluted	10 fold diluted		Undiluted		10 fold diluted		
					Replicates	Mean	Replicates	Mean	
1	Negative feed matrix	50.00	50.00	Negative	50.00		50.00		Negative
					50.00	50.00	50.00	50.00	
2	0.5 % bovine MBM	24.17	27.79	Positive	26.16		32.36		Positive
					25.34	25.75	31.19	31.78	
3	0.5 % ovine MBM	40.20	45.96	Negative	50.00		50.00		Negative
					42.35	46.18	50.00	50.00	
4	0.5 % porcine MBM	49.32	50.00	Negative	46.33		50.00		Negative
					46.16	46.25	50.00	50.00	
5	0.5 % avian MBM	50.00	50.00	Negative	50.00		50.00		Negative
					50.00	50.00	50.00	50.00	
6	0.5 % fishmeal	50.00	50.00	Negative	50.00		50.00		Negative
					50.00	50.00	50.00	50.00	
7	0.5 % bovine MBM + 0.5 % porcine MBM	24.08	27.08	Positive	26.63		32.14		Positive
					27.68	27.16	34.17	33.16	
8	0.5 % ovine MBM + 0.5 % avian MBM	50.00	44.65	Negative	38.85		50.00		Negative
					45.02	41.94	50.00	50.00	
9	0.5 % avian MBM + 0.5 % fishmeal	50.00	50.00	Negative	50.00		50.00		Negative
					50.00	50.00	50.00	50.00	
10	Negative feed matrix	50.00	50.00	Negative	50.00		50.00		Negative
					50.00	50.00	50.00	50.00	

*** Ct values < 40 cycles are considered as significant**

Table 9.5. PCR results obtained with the CRA-W PCR target on DNAs produced from the VLA sample set with the phenol/chloroform extraction method of the CRA-W

Sample	Animal protein content	Ct values obtained with CRA-W extracts*				Conclusion
		10 fold diluted		100 fold diluted		
		Replicates	Mean	Replicates	Mean	
1	Negative feed matrix	50.00		50.00		Negative
		50.00	50.00	50.00	50.00	
2	0.5 % bovine MBM	50.00		27.06		Positive
		50.00	50.00	27.97	27.52	
3	0.5 % ovine MBM	50.00		41.97		Negative
		50.00	50.00	43.69	42.83	
4	0.5 % porcine MBM	50.00		50.00		Negative
		50.00	50.00	50.00	50.00	
5	0.5 % avian MBM	50.00		50.00		Negative
		50.00	50.00	38.48	44.24	
6	0.5 % fishmeal	50.00		50.00		Negative
		50.00	50.00	50.00	50.00	
7	0.5 % bovine MBM + 0.5 % porcine MBM	50.00		27.81		Positive
		50.00	50.00	27.61	27.71	
8	0.5 % ovine MBM + 0.5 % avian MBM	50.00		50.00		Negative
		50.00	50.00	50.00	50.00	
9	0.5 % avian MBM + 0.5 % fishmeal	50.00		50.00		Negative
		50.00	50.00	50.00	50.00	
10	Negative feed matrix	50.00		50.00		Negative
		50.00	50.00	50.00	50.00	

*** Ct values < 40 cycles are considered as significant**

Table 9.6. PCR results obtained with the VLA bovine PCR test on DNAs produced with VLA and the CRA-W extraction methods from the VLA sample set

Sample	Animal protein content	Ct values obtained with VLA extracts*	Conclusion	Ct values obtained with CRA-W extracts*		Conclusion
				Replicates	Mean	
1	Negative feed matrix	40,00	Negative	40,00		Negative
				40,00	40,00	
2	0.5 % bovine MBM	27,52	Positive	33,68		Positive
				33,64	33,66	
3	0.5 % ovine MBM	40,00	Negative	40,00		Negative
				40,00	40,00	
4	0.5 % porcine MBM	39,18	Negative	40,00		Negative
				40,00	40,00	
5	0.5 % avian MBM	40,00	Negative	40,00		Negative
				40,00	40,00	
6	0.5 % fishmeal	40,00	Negative	40,00		Negative
				40,00	40,00	
7	0.5 % bovine MBM + 0.5 % porcine MBM	26,82	Positive	35,77		Positive
				35,57	35,67	
8	0.5 % ovine MBM + 0.5 % avian MBM	40,00	Negative	40,00		Negative
				40,00	40,00	
9	0.5 % avian MBM + 0.5 % fishmeal	40,00	Negative	40,00		Negative
				40,00	40,00	
10	Negative feed matrix	40,00	Negative	40,00		Negative
				40,00	40,00	

*** Ct values < 33,67 cycles are considered as significant**

Table 9.7. PCR results obtained with the CRA-W PCR target on DNAs produced with the VLA and the CRA-W extraction methods from the CRA-W sample set.

Sample	Animal protein content	Ct values obtained with VLA extracts*		Conclusion	Ct values obtained with CRA-W extracts*				Conclusion
		Undiluted	10 fold diluted		Undiluted		10 fold diluted		
					Replicates	Mean	Replicates	Mean	
1	Negative feed matrix	50,00	50,00	Negative	50,00		50,00		Negative
					39,62	44,81	50,00	50,00	
2	0.0033 % bovine MBM	42,26	50,00	Negative	49,57		50,00		Negative
					50,00	49,79	50,00	50,00	
3	0.0075 % bovine MBM	48,75	50,00	Negative	40,92		50,00		Negative
					50,00	45,46	50,00	50,00	
4	0.0125 % bovine MBM	50,00	40,56	Negative	39,33		50,00		Positive
					38,56	38,95	50,00	50,00	
5	0.0250 % bovine MBM	38,61	50,00	Positive	38,16		50,00		Positive
					36,70	37,43	45,63	47,82	
6	0.0500 % bovine MBM	40,28	50,00	Negative	36,62		41,84		Positive
					36,37	36,50	50,00	45,92	
7	0.0750 % bovine MBM	37,47	39,55	Positive	36,57		40,74		Positive
					36,49	36,53	40,91	40,83	
8	0.1000 % bovine MBM	38,43	50,00	Positive	34,97		39,76		Positive
					35,76	35,37	41,58	40,67	
9	0.1000 % bovine MBM	39,00	42,21	Positive	37,84		44,46		Positive
					36,37	37,11	38,91	41,69	
10	0.2000 % bovine MBM	37,87	50,00	Positive	35,16		38,73		Positive
					35,62	35,39	38,89	38,81	

*** Ct values < 40 cycles are considered as significant**