final report

Project Code: A.TSE.0012

Prepared by: Zhigang Guo, Bronwyn Murphy & Kerry R Emslie
National Measurement Institute

Date published: July 2005

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

The Detection of Animal Derived DNA in Stockfeed Existing Test Improvement and Screening Test Evaluation

This is an MLA Donor Company funded project.

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government and contributions from the Australian Meat Processor Corporation to support the research and development detailed in this publication.

Contents

ABSTRACT

The practice of feeding meat and bone meal (MBM) to cattle has been banned following its implication in the spread of Bovine spongiform encephalopathy (BSE) through the cattle population. Australia is in a very low risk category for BSE in cattle. However, to maintain this status and ensure the ban on feeding ruminant derived materials to cattle and sheep is implemented, the ability to detect animal-derived tissued in stockfeed is considered critical. A polymerase chain reaction (PCR) technique has previously been developed that detects the presence of ruminant DNA extracted from MBM. A Reporting Threshold was established such that 95% of a series of stockfeed samples spiked with either 0.5% (w/w) rendered bovine or ovine tissue would be reported as positive subject to confirmatory analysis. This study involved further validation of the PCR screen by assessing its reactivity against stockfeed ingredients that are excluded from the current Australian ban such as tallow, gelatin and milk powder and against MBM that has been rendered to the stringent conditions required of the European Union (EU). Rapid screening tests for detecting animal protein in stockfeed have also been evaluated and a procedure developed to monitor for inhibitory substances in stockfeed that could give rise to a false negative result.

EXECUTIVE SUMMARY

A protocol for the identification of rendered bovine and ovine material in stockfeed has been validated. The primary purpose of the method is to monitor for inadvertant contamination of stockfeed with ruminant material during the production process. The rendered material is detected using a polymerase chain reaction (PCR) assay that has been designed, specifically, to amplify a conservative region of the cytochrome b gene in ruminant species. The variable nature of meat and bone meal (MBM) components such as animal species and tissue type together with the range of rendering treatments and processes makes quantitative analysis of MBM in stockfeed extremely difficult.

This method has been validated for the qualitative detection of rendered ovine or bovine material in stockfeed. The method comprises an initial real-time PCR screen together with melt curve analysis to identify presumptive positive samples. Samples that are presumptive positive based on the initial screen should undergo confirmatory DNA sequence analysis to identify the species present and confirm the presence of ruminant material.

The method sets a threshold cycle in the PCR whereby samples with a C_T value below the threshold cycle will be reported as positive provided this is supported by the expected melt curve analysis data and confirmatory analysis. The Reporting Threshold cycle was established based on analysis of a range of plant-based stockfeed samples spiked with either 0.5% (w/w) rendered ovine or bovine material (n = 9). The Reporting Threshold was set so that the probability of a stockfeed sample spiked with 0.5% (w/w) rendered ovine or bovine material falling below the PCR threshold cycle or the Reporting Threshold is 95%. The threshold level of 0.5% (w/w) MBM was set as being fit-for-the-purpose of the assay and is not the Limit of Detection (LOD).

Because the method is DNA-based and not tissue-specific, it will detect DNA from ovine and bovine material apart from MBM, provided sufficient DNA is present in the tissue. For instance, the method detects DNA from bovine milk powder (depending on the type of milk powder sample) when spiked at a level of 5% (w/w) in stockfeed.

Rapid screening tests for detecting animal protein in stockfeed have been evaluated. Whilst these tests are significantly cheaper than the PCR screen, they were less sensitive than the PCR assay and interpretation of results is subjective at the LOD. Thus they are not suitable for use as a confirmatory analysis following a PCR screen.

Note: The practice of feeding MBM, the cattle feed made from the rendered remains of dead animals, to cattle has been implicated in the spread of BSE through the cattle population. A safe level of rendered meat in stockfeed that will exclude transmission of BSE has not been established. Presence of rendered bovine or ovine material in stockfeed below the Reporting Threshold does not exclude the possibility that BSE could be transmitted via that stockfeed.

BACKGROUND

Bovine spongiform encephalopathy (BSE) is a fatal degenerative disease affecting the central nervous system of cattle. The disease was first reported in the UK in 1986, with up to 100,000 cattle affected by 1993. The practice of feeding meat and bone meal (MBM) to cattle has been implicated in the spread of BSE through the cattle population (1). More than 80 cases of a variant of the fatal Creutzfeldt-Jakob disease (nvCJD), which affects humans, have been reported in the UK. This human disease has been linked etiologically to BSE (2).

In 1996, the World Health Organisation made a recommendation that ruminant protein should not be included in ruminant feeds. This was implemented as a preventative measure to avoid the spread of BSE and to minimise transmission of BSE from bovines to humans. In March 2001, the Agricultural and Resource Management Council of Australia and New Zealand extended this ban in Australia to include all animal material (excluding gelatine, tallow and milk).

In 2000, the Scientific Steering Committee advising the European Commission on BSE-related issues rated Australia in the lowest risk category for having cattle infected with the BSE agent (GBR level 1). However, an important component of the risk management measures required for Australia to maintain such a status is the ability to monitor and audit for the presence of animal-derived tissue in stockfeed.

Compliance with Australia's ruminant feed ban needs to be monitored at a number of levels from imported stockfeed through to domestic stockfeed production. In each case, it is desirable to have a testing regime that is 'fit-for-purpose' as the testing requirements for imported stockfeed differ slightly to those for domestic stockfeed. Currently, the only animal material that may be fed to ruminants is tallow, gelatin and milk or milk products of Australian or New Zealand origin. Importation of animal-derived stockfeed material, other than fishmeal, is currently only permitted from New Zealand.

Further considerations when designing a test protocol are the level of expertise required and the cost of testing (3). DNA-based methods must be conducted in a laboratory by highly trained staff. On the other hand, commercial lateral flow strips assays for detecting either mammalian or ruminant material in stockfeed are capable of detecting down to 1% (w/w) animal material in stockfeed according to the manufacturers. This is a similar level of sensitivity to that currently obtained using the PCR method. These lateral flow strip assays do not require specialised instrumentation and have the potential to be used in the field.

This study involved further validation of the PCR method previously developed by the Australian Government Analytical Laboratories (AGAL) (4). The assay was assessed against stockfeed ingredients that are excluded from the current Australian ban such as tallow, gelatin and milk powder and against MBM that has been rendered to the stringent conditions required of the European Union (EU). Rapid screening tests for detecting animal protein in stockfeed have been evaluated and a procedure developed to monitor for inhibitory substances in stockfeed that could give rise to a false negative result.

OBJECTIVES

The major objectives of this study were:

- to further validate the PCR method previously developed at AGAL by analysing animal-derived stockfeed ingredients such as tallow, gelatin and milk powder and ruminant material subjected to the EU rendering conditions,
- to evaluate a rapid screening test for the detection of animal material in stockfeed, and
- to develop a procedure to monitor for inhibitory substances in the stockfeed that could give rise to a false negative result.

METHODOLOGY

Stockfeed Ingredients

A range of stockfeed ingredients such as MBM, tallow, gelatin and milk powder were sourced through the Office of the Chief Veterinary Officer, Product Integrity Animal and Plant Health, Department of Agriculture Fisheries and Forestry (*Table AI*). MBM included two ovine MBM samples that had been rendered to the conditions required by the EU and one bovine MBM sample rendered using a new system that involves an alkali hydrolysis step.

A range of stockfeed samples were analysed including samples sourced through Meat and Livestock Australia and commercial samples submitted to the National Measurement Institute (NMI) for analysis. Samples of stockfeed pellets (10 – 100 g) were homogenised (Vorwerk, Thermomix) and pulsed at level 9 for 30 seconds to form a powder prior to analysis.

Preparation of spiked stockfeed standards

A series of plant-based stockfeed standards containing 0.5% (w/w) – 10% (w/w) ovine MBM was prepared. Plant-based stockfeed standards containing 5% (w/w) and 10% (w/w) milk powder were prepared using both a buttermilk powder sample (M1 2987870) and a skim milk powder sample (M7 2934439). Since ruminant DNA was not detected in samples of 100% tallow or 100% gelatin, stockfeed standards spiked with lower concentrations of these two ingredients were not prepared.

Detection of animal DNA using PCR

DNA Extraction

DNA was extracted from two tallow samples using a DNA extraction technique specifically designed for high fat samples whilst DNA from all other samples was extracted using the standard NMI method for stockfeed samples. The standard NMI method is designed to isolate fragmented DNA of sizes 100 base pairs (bp) to 10,000 bp, as the rendering process is known to degrade DNA. For the standard method, DNA was extracted from samples (450-500 mg) using the Wizard Genomic DNA Extraction Kit (Promega) and purified using the QIAquick® PCR column purification kit (Qiagen). Both kits were used in accordance with Manufacturer's instructions with slight modifications. DNA was stored at –20^{o}C prior to PCR amplification.

The DNA concentration in extracted samples was estimated by measuring UV absorbance at 260 nm and the DNA quality was assessed using the 260 nm to 280 nm UV absorption ratios (Gene Quant Spectrophotometer, Pharmacia Biotech, Cambridge UK). Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9 whilst samples with protein contamination have a lower ratio (Ref: Dneasy Plant Mini Kit Handbook, Qiagen).

Real-time PCR analysis

Target DNA was amplified by real-time PCR (ABI 7700 Sequence Detection System) using the standard NMI method for detecting ruminant DNA in stockfeed (Method RD28) that had previously been developed as part of Meat and Livestock Australia (MLA) Project TSE.003.

In general, the sample DNA extract is analysed by PCR in duplicate using two different amounts of template DNA, 8 and 40 ng DNA. Analysis with 40 ng template DNA is used to monitor for the presence of PCR inhibitors in the DNA extract. The PCR amplification reaction volume of 20 μL included 10 μL 2xSYBR Green 1 Mastermix (Applied Biosystems, U.S.A.), 500 nM of both forward and reverse primers (Geneworks, Australia) and either 8 or 40 ng genomic DNA. The reactions were each performed within 96-well PCR plates with automated detection. The thermal cycling conditions used were as follows: 1 step of 95^{o}C for 10 min followed by 40 cycles comprising two steps of 95^{o}C for 15 s and 60^{o}C for 1 min. Data was analysed using SDS Ver. 1.9 (Applied Biosystems, U.S.A).

SYBR Green, a dye that fluoresces when bound to double-stranded DNA, is present in the reaction mix and the amount of PCR product in the sample is determined by measuring the level of fluorescence accumulation in real time. When the fluorescence reaches a defined level this is referred to as the Threshold Cycle (C_T). The C_T value is calculated for each sample and is dependent on the amount of target DNA in the initial sample. Hence, a sample with a large amount of target DNA would require fewer cycles to reach the C_T than a sample with a very small amount of target DNA. When no amplification has occurred, reporter fluorescence is not significantly different to the baseline signal level.

Melt Curve analysis

Every PCR product has a particular temperature at which the two DNA strands will separate or 'melt' and this characteristic is dependent on both the length of the PCR product and the sequence. To determine the melt curve, a sample is slowly heated to 95°C and the temperature at which the PCR product melts is determined.

Melt curve analysis was performed immediately following PCR. The thermal profile comprised the following three steps: 95^{o}C for 15 s, 60^{o}C for 20 s then 95^{o}C for 15 s. Data was collected between the second and third steps where the ramp time was set at 20 min.

Defining Reporting Threshold Cycle in PCR screen

Samples containing no ruminant DNA have a high C_T value (>31) whilst samples containing ruminant DNA have a lower C_T value. For example, a sample of fresh bovine tissue will generally give a C_T value <19.

The Reporting Threshold cycle is the threshold cycle in the PCR whereby samples with a C_T value below the threshold cycle are considered as 'presumptive positive' provided this is supported by melt temperature data. The Reporting Threshold was established based on analysis of a range of plant-based stockfeed samples spiked with either 0.5% (w/w) rendered ovine or bovine material (n = 9). The Reporting Threshold was set at 29.6 and 27.1 for 8 ng and 40 ng DNA templates, respectively, which are 2 standard deviations from the mean in each case.

DNA Sequence Analysis

DNA sequence analysis of PCR products was undertaken on an ABI3100 Genetic Analyzer. At least 55 bases of readable sequence from the internal 80 bp portion of the PCR product were required for data interpretation. For positive identification of bovine or ovine material in the sample, the readable sequence must match the relevant bovine or ovine sequence in the Genbank® database.

Detection of animal protein using lateral flow strips

Lateral Flow strip assays are simple, quick immunochromatographic assays that do not require specialised equipment and are suitable for on-site or field testing. The lateral flow strip contains three parts- the top absorbent pad, the test area and the bottom absorbent pad. The bottom absorbent pad is placed into the sample creating capillary flow of sample up through the test strip area into the top absorbent pad. The test strip contains coloured particles conjugated to antibodies specific to target proteins. These antibody/coloured particle complexes bind to target proteins in the sample and then travel up the strip to the test area. Additional antibodies specific to the target protein are immobilised in a zone within the test area and these immobilised antibodies bind the complex, causing the complexes to concentrate and a coloured line develops.

All test strips have a control zone in addition to the test zone. If the control zone does not develop within the incubation time, the test strip is invalid and the sample should be tested with another strip. Provided that the bottom and top absorbent pads are cut away from the test strip within one hour after completion of the test, the test strip may be archived.

Neogen 'Agriscreen for Ruminant Feed' lateral flow strips

The Neogen 'Agriscreen for Ruminant Feed' lateral flow strips are used for the qualitative detection of ruminant by-products in animal feed. The manufacturer claims the strips can detect as little as 1% (w/w) ruminant by-products in feed and feed supplements. These strips were used in accordance with manufacturer's instructions.

Briefly, sample (1 g) was placed into a sterile 50 mL conical tube together with Extraction Additive (0.2 g) and Extraction Solution (10 mL). The solution was mixed thoroughly by shaking and then heated in a boiling water bath for 10 minutes. The tube was removed from the heat source, mixed and then sample extract (0.5 mL) transferred to an Eppendorf tube. A lateral flow strip was then placed into the Eppendorf tube ensuring that the bottom absorbent pad was in the sample and then left for 10 minutes before reading results.

If a line of any intensity forms in the test zone and another line forms in the control zone (2lines in total) the sample is positive (*Figure 1*). It is not necessary to wait a full 10 minutes if 2 lines develop. If there is no visible line in the test zone, but a visible line in the control zone, the sample is negative. If there is no line in the control zone, the test strip is invalid and the sample should be re-tested with another test strip.

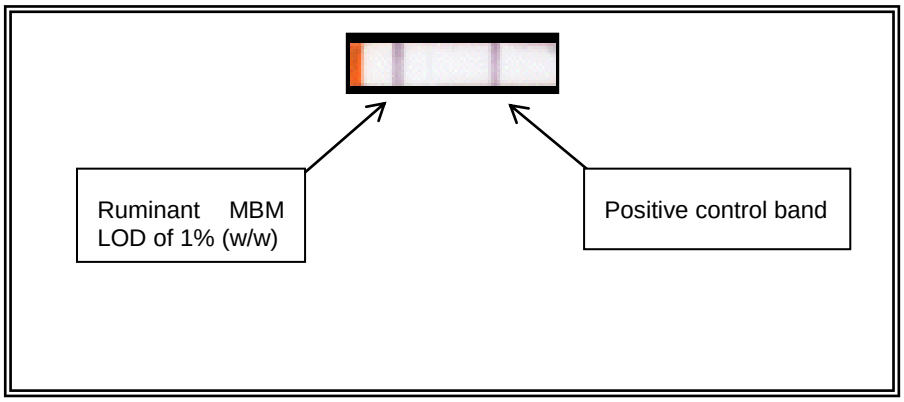

Figure 1: Identification of bands on the Neogen 'Agriscreen for Ruminant Feed' lateral flow strips

Strategic Diagnostics FeedChek™ MBM Test kit lateral flow strips

The Strategic Diagnostics (SDI) FeedChek™ MBM Test kit lateral flow strips are used for the qualitative detection of mammalian and avian protein in animal feed. Each strip incorporates two tests. According to the manufacturer, one test indicates the presence of mammalian, avian and fish MBM down to 0.1% (w/w) and is directed to connective tissue proteins.

The other test indicates the presence of mammalian MBM (MMBM) at or above 1% (w/w) in the sample and is directed against less prevalent muscle proteins that are mammal-specific. These strips were used in accordance with manufacturer's instructions. Briefly, a level plastic spoon of feed sample is placed into extraction buffer in a closed container and then shaken for 15 seconds. A FeedChek MBM test strip is then placed into the cup with the arrows pointing down and then left for 10 minutes before reading results.

The presence of one line (control line) on the membrane indicates a negative sample. The presence of 2 lines indicates that the sample is positive for mammalian, avian and/or fish connective tissue protein (≥ 0.1%). The presence of 3 lines indicates that the sample is positive for mammalian muscle protein (≥ 1.0%) (*Figure 2*).

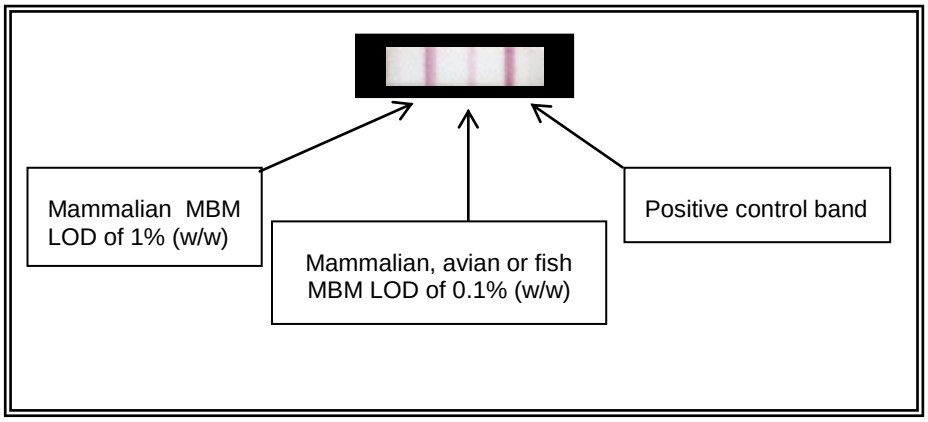

Figure 2: Identification of bands on the SDI FeedChek™ MBM Test kit lateral flow strips

RESULTS AND DISCUSSION

Detection of animal DNA using PCR

DNA extracts from rendered MBM were relatively pure based on the absorbance ratio of 260 nm/280 nm (Table AII). On the other hand, both the yield and level of purity of DNA extracted from gelatin, tallow and milk powder were lower than that found for rendered MBM.

Detection of MBM

The Reporting Threshold that has previously been set for the PCR test was based on analysis of a range of plant-based stockfeed samples that had been spiked with 0.5% (w/w) bovine MBM. However, the bovine MBM used to prepare these samples had not been rendered to the conditions specified by the EU. To examine the sensitivity of the PCR to detect MBM rendered to EU conditions, standards were prepared using ovine MBM rendered to EU conditions.

Using the standard Reporting Threshold for this method, ruminant DNA was detected in both samples of 100% ovine MBM rendered to EU conditions. The average C_T values for the 8 ng DNA template from these samples were 18.93 and 19.51 indicating a very strong positive result in both cases. Analysis of stockfeed standards spiked with ovine MBM showed that the PCR is capable of detecting down to 0.5% (w/w) ovine MBM using 8 ng template DNA (*Table AIII*).

In a recent intercomparison study conducted by the European Commission's Joint Research Centre the PCR screen did not detect the presence of Processed Animal Protein (PAP) in the intercomparison samples (*Table AV*). Samples from the intercomparison study had a maximum amount of 0.25% (w/w) ruminant PAP (0.5% (w/w) of a 1:1 mix of pork and beef was used in preparation of the PAP material). Thus the samples in the intercomparison study were spiked with PAP at levels below the current Reporting Threshold for this PCR Method.

A sample of bovine MBM rendered using a new alkaline rendering process was detected with a similar level of sensitivity to the sample of ovine MBM that had been rendered to the EU conditions (*Table AIII*).

Detection of gelatin, tallow and milk powder

A DNA extract (8 ng) from the gelatin sample gave a C_T value of 33.05 and the 7 tallow samples returned C_T values ranging from 36.45-45.00 (*Table AIII*). Hence ruminant DNA was not detected in either the gelatin sample or any of the tallow samples.

C_T values for DNA extracted from 100% milk powder ranged from 18.76 to 26.06 indicating a positive result for all samples. The buttermilk powder samples had the lowest C_T values indicating a strong positive result for these samples whilst the skim milk powder samples had slightly higher C_T values. Analysis of stockfeed samples spiked with milk powder indicated that stockfeed containing 5% (w/w) milk powder may be detected using the PCR method depending to some extent on the type of milk powder in the sample (*Table AIII*).

Detection of non-ruminant species

Over the last 20 months, NMI has analysed a number of commercial fishmeal samples using the PCR method to detect the presence of ruminant material. On one occasion, sample Comm121, DNA from a fish species was amplified using the PCR and the melt curve was similar to that for the bovine PCR product. DNA sequence analysis revealed that the PCR product most closely matched the fish species *Scomber australasicus* and Spanish mackerel. This example highlights the importance of additional analysis to confirm the presence of animal material. Whilst melt curve analysis is useful for detecting some non-specific amplification products, it cannot be used as a confirmatory step by itself. In addition, even though the PCR screen has been designed to

detect ruminant species, it is not possible to verify selectivity against all other species. Sequence analysis of the final PCR product is the only robust method for identifying the individual animal species that has given rise to the PCR product.

Melt Curve Analysis of PCR products

The melt temperature (T_m) for the bovine PCR product from the 0.5% RM was 80.09 ± 0.16°C using 40 ng DNA template and was slightly higher at 80.36 ± 0.22°C using an 8 ng DNA template (n = 12). The T_m for the ovine PCR product from the PSF RM was 78.57 ± 0.22°C and 78.91 ± 0.25°C (n = 11) for the 40 ng and 8 ng DNA templates, respectively. Non-specific primer-dimers have a melt curve that is very broad with a peak between 70-75°C.

Factors that may affect result from PCR assay

The presence of inhibitory substances in stockfeed and non-specific amplification were considered as two factors that could give rise to an incorrect result in the PCR screen. To identify additional factors, a risk analysis of the PCR screen was undertaken (*Table AIV*).

Monitoring for PCR inhibitors in the DNA extract

When the PCR method was initially developed as part of Project TSE.003, there was concern regarding the possibility that inhibitory substances in the stockfeed could give rise to a false negative result. In order to investigate this possibility, samples were routinely analysed using two different amount of template DNA (8 ng and 40 ng DNA). The difference in C_T values (ΔC_{Tdiln}) was then used to identify samples that may contain inhibitory substances.

Theoretically, if a PCR is amplifying with 100% efficiency, the ΔC_{Tdiln} when one assay has twice the starting DNA template as the other (eg. 2 ng and 4 ng DNA) is exactly 1 C_T value. When one assay has five times the starting DNA template as the other assay (eg. 8 ng and 40 ng DNA) the theoretical ΔC_{Tdiln} will be 2.32 when the efficiency of both reactions is 100% (*Table I*).

The ΔC_{Tdiln} between the two assays will increase as the amplification efficiency of the reactions decreases. In contrast, if inhibitors are present in the DNA extract the ΔC_{Tdiln} for the two assays would be expected to decrease. This decrease occurs because the inhibitory effect is more pronounced on the 40 ng DNA assay since the concentration of inhibitors is higher in this sample. For example if the PCR from the 40 ng DNA template is inhibited by 10%, but the PCR from the 8 ng DNA template is only inhibited by 5%, then the theoretical ΔC_{Tdiln} for the two assays will be 1.31 (*Table I*).

Table I. Effect of amplification efficiency on theoretical ΔCT_{diln} for two assays starting with 8 ng and 40 ng DNA template

Efficiency		ΔC_{Tdiln}
8 ng template	40 ng template	
100%	100%	2.32
95%	95%	2.41
90%	90%	2.51
80%	80%	2.74
95%	90%	1.31
95%	85%	0.09

Performance criteria for the Inhibition Control were set based on analysis of commercial assays (n=116). There was a strong correlation (R^2 = 0.942) between CT values for a sample when using 40 ng and 8 ng DNA templates (*Figure 3*). The mean ΔCT_{diln} for these samples was 2.36 ± 1.01 (*Figure 3*) which is very close to the theoretical value at 100% efficiency of 2.32 (*Table I*). This indicates that there is little evidence of inhibitory compounds in the DNA extracts from commercial samples and also demonstrates that analysis of samples at two dilutions is a suitable approach to monitoring PCR inhibition.

The Performance Criterion for monitoring inhibition was set such that the sample was considered to show signs of inhibition if the ΔCT_{diln} value was less than 0.34 which is 2 standard deviations from the mean of 2.36.

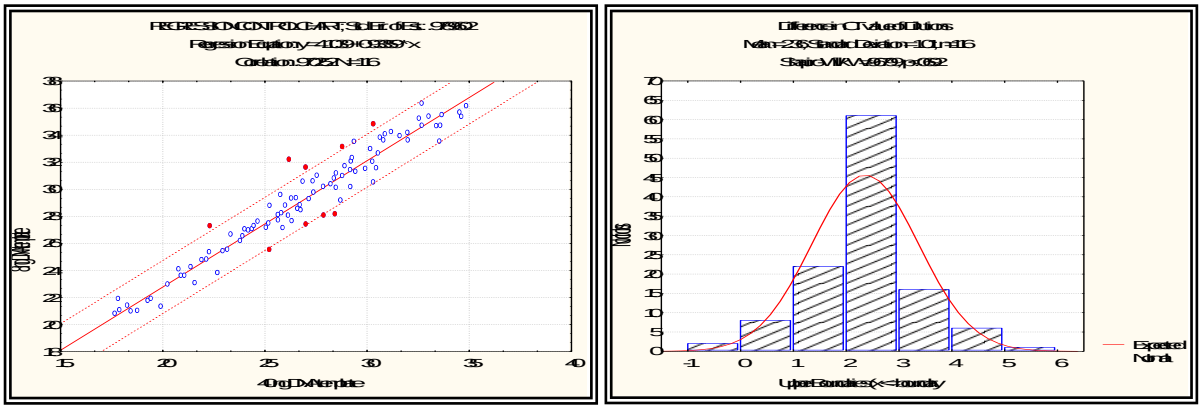

Figure 3: PCR Analysis and distribution of ΔCT_{dupl} values for commercial samples (n=116)

Repeatability of PCR screen CT values

Results from homogeneity analysis of PSF RM and the 0.5% RM Reference Materials were used to assess the repeatability of the PCR screen CT values (*Table II*). The Relative Standard Deviation (%RSD) for the 0.5% RM with an average CT value of 25.03 was 24%. The %RSD for the PSF RM with an average CT value of 30.32 was higher at 43%.

Table II. Homogeneity of Reference Materials

Value	Reference Material	Mean	SD	%RSD	S_a	S_s	F	p-value
CT value								
	PSF RM	30.32	0.60	1.98	0.59	0.10	1.06	0.463
	0.5% RM	25.03	0.33	1.33	0.18	0.29	6.30	0.004
Nominal amount of target DNA								
	PSF RM	0.015	0.007	42.60	0.007	0.001	1.02	0.482
	0.5% RM	0.57	0.14	24.15	0.08	0.116	5.46	0.007

S_a, Estimate of analytical standard deviation from ANOVA; S_s, Estimate of sampling standard deviation from ANOVA

The repeatability of a procedure can also be assessed by examining the difference between duplicate analyses on identical samples (Fig. 4). A ΔCT_{dupl} of 0.62 equates to a %RSD for the analyte of 30%. Approximately 90% of samples with a CT value below 31 have an RSD <30. The repeatability of the analysis is reduced for samples with a CT value above 31.

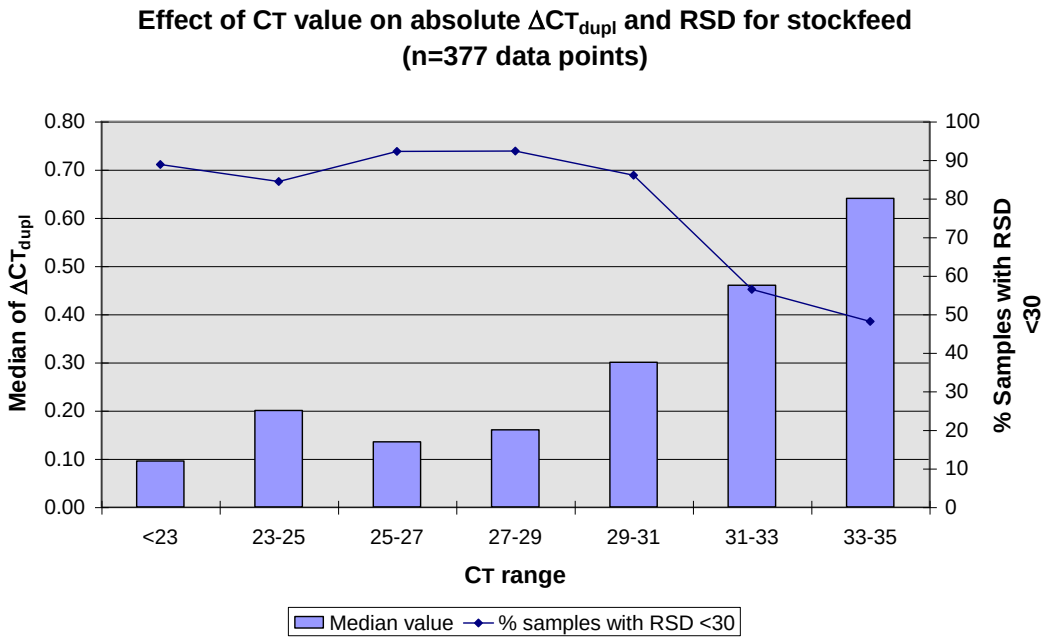

Figure 4: The effect of CT values on RSD of sample replicates. The median value of the absolute difference in CT value for duplicate analyses is depicted in the histograms. The solid line indicates the % samples with a $\%RSD_{DNA}$ of <30%.

Detection of animal protein using lateral flow strips

A series of samples have been analysed using the Neogen 'Agriscreen for Ruminant Feed' and the SDI 'FeedChek™ MBM Test' lateral flow strips. These include in-house MBM standards, blind samples previously provided by the MLA, samples from the European Commission's Joint Research Centre intercomparison study, samples collected as part of the 'Australian Ruminant Feed Ban Compliance Test' and commercial samples. The majority of samples were analysed in duplicate and results were scored by visual inspection of the strip.

Evaluation of in-house standards and EU intercomparison study samples

Based on analysis of in-house standards and samples from the European Commission's Joint Research Centre intercomparison study, the lateral flow strip assays did not give rise to any false positives when analysing plant-based stockfeed (*Table AV*; see PSF RM, SF B-E; EU Sample MAT1). However, the Neogen strips only detected 2 of the 5 in-house plant-based stockfeed standards spiked with 0.5% (w/w) bovine MBM in stockfeed although all five spiked standards had returned a positive result previously using PCR analysis (*Table AV*). Whilst 0.5% (w/w) spiked standards were detected by the more sensitive SDI FeedChek MBM test that is specific for mammalian, avian or fish connective tissue protein, they were not detected using the mammalian MBM specific test.

MBM of mammalian, avian or fish origin was detected using the SDI strips in all EU intercomparison samples containing as little as 0.1% (w/w) PAP from beef and pork (*Table AV*). Faint bands were also visible corresponding to the SDI mammalian-specific muscle protein test

in all three samples containing 0.5% (w/w) PAP from beef and pork and two of these samples were just detected using the Neogen test.

The in-house standards containing 4% (w/w) ovine MBM (rendered to EU conditions) was detected as a faint band using the Neogen test but the band associated with 1% (w/w) ovine MBM (rendered to EU conditions) was barely visible. The 4% (w/w) ovine MBM standard was not detected using the SDI strips. In all cases apart from the 100% rendered ovine MBM, the band that relates to the presence of ruminant material is much weaker than the positive control band.

Evaluation of commercial stockfeed and fishmeal samples

A sub-set of the stockfeed samples obtained through MLA for Project TSE.003 was analysed using the lateral flow strips (Table AV). The 'Broiler Chicken' feeds contained 5.5 - 7.75% (w/w) MBM derived predominantly from ovine and bovine sources. Ruminant material was detected in 4 of the 5 Broiler Chicken feeds analysed using the Neogen lateral flow strips. Results for Broiler feeds containing 5.75% (w/w) MBM and 2.5-2.75% (w/w) poultry meal (127-78 and 127-91) were either very weak or not detected using the Neogen test despite these samples returning a relatively strong positive result in the PCR analysis (see Table 3. Final Report TSE.003). Since the precise composition of the MBM is unknown in these samples, it is possible that the amount of bovine/ovine MBM was significantly below 5% (w/w) in the Broiler chicken feeds. Alternatively, this result may indicate a matrix effect whereby certain non-ruminant stockfeed ingredients could be reducing the sensitivity of the Neogen antibody-based assay.

Five 'Dairy Cattle' feeds containing no meat meal were analysed using the lateral flow strips. All of these samples had previously been analysed using the PCR technique (see Table 3. Final Report TSE.003). In agreement with the PCR results, all Dairy Cattle feeds were negative for ruminant MBM using the Neogen kit.

Several commercial stockfeed and fishmeal samples were selected for analysis using the Neogen lateral flow strips (*Table AV*). All of these samples had previously been analysed using the PCR technique. Ruminant material was not detected in 2 of the plant-based stockfeed samples (Comm151 and Comm152) using the Neogen test and this is in agreement with the PCR results. Three plant-based stockfeed samples (Comm9, Comm91 and Comm41) tested positive for ruminant material using the PCR method and were further analysed by DNA sequencing to identify the species present in the samples. One sample contained ovine material, one bovine material and the third sample contained a mixture of both ovine and bovine material. Ruminant and mammalian material was not detected in these three samples using the Neogen and SDI lateral flow strips, respectively. This suggests that, at least for these samples, the PCR method is more sensitive than the Neogen or SDI lateral flow strips.

Four fishmeal samples (Comm111, Comm112, Comm123 and Comm114) that tested positive using the PCR method were further analysed using DNA sequencing and shown to contain either ovine or bovine material (*Table AV*). Ruminant material was detected in all four of these samples using the Neogen lateral flow strips and mammalian material was detected in the two samples analysed using the SDI kit (Comm112 and Comm111). A fishmeal sample (Comm121) that did not test positive using the PCR method was also negative using the lateral flow strips although all fishmeal samples showed a positive result for the MBM test that detects mammalian, avian and fish connective tissue protein, as expected. Unfortunately, the level of contamination in these samples is unknown as they are from commercial origin.

Evaluation of milk powder, gelatin and tallow

Samples of 100% tallow and 100% buttermilk, skim milk and whey milk powder were not detected using the lateral flow strips nor were samples of 5% (w/w) buttermilk or skim milk powder in plant-based stockfeed (data not shown). The gelatin sample was difficult to evaluate using the Neogen kit as the method requires a boiling step which resulted in the gelatin solution becoming very viscous. Because of the viscosity of the solution it did not migrate up the lateral flow strip and hence had no opportunity to react with the antibodies on the strip. In the case of the SDI kit, the result from gelatin was surprising in that the MMBM band was stronger than the MBM band for both the 100% and 1% (w/w) gelatin samples.

Factors that may affect result from lateral flow strip assays

A number of potential factors that may affect results from lateral flow strips were identified (*Table AVI*). Of particular importance is the need to read lateral flow strips within 30 minutes of completing the assay. For archiving purposes, the strip pads either side of the test strip should be removed within one hour of test completion since faint bands can appear with time if the complete lateral flow strip is left intact and this may give rise to a false positive result.

CONCLUSION

The PCR method together with melt curve analysis of the PCR product has been validated as a screen for detection of bovine and ovine material in stockfeed. Since the PCR method is DNA-based, it is not tissue specific and may detect milk powder in stockfeed when present at a level of 5% (w/w). The PCR screen did not detect tallow or gelatin samples. Presumptive positive results from the PCR screen should undergo DNA sequence analysis to identify the animal species present and confirm the presence of ruminant material.

The Neogen lateral flow strips are capable of detecting down to 1% (w/w) ruminant material in stockfeed as claimed by the manufacturer. However, in our experience, even with 5-7% (w/w) MBM the bands are faint and much weaker in intensity than the control band. The SDI test that detects mammalian muscle protein has a similar level of sensitivity to that of the Neogen strip that detects ruminant protein. The SDI mammalian-specific test has one disadvantage over the Neogen test in that it detects mammalian tissue rather than the more selective Neogen test that is claimed to only detect ruminant material. However, sample preparation requirements for the SDI kit are much simpler than for the Neogen kit.

The Neogen assay had no false positives on analysis of a range of stockfeeds including plant-based stockfeeds, fishmeal and a stockfeed sample containing 5% (w/w) poultry meal although it did cross-react with samples of 100% fresh pork or chicken (data not shown). The SDI had no false positives with the limited number of analyses undertaken.

Both the Neogen and SDI lateral flow strips are significantly cheaper that the PCR method. However, since they are less sensitive than the PCR assay, they are not suitable for use as a confirmatory analysis following a PCR screen.

REFERENCES

1. Ainsworth C. 2000. A Killer is born. New Scientist. 2263:7

2. Mackenzie D. 2000. Mad Meat. New Scientist. 2263:8-9

3. Gizzi G, van Raamsdonk LW, Baeten V, Murray I, Berben G, Brambilla G, von Holst C. 2003. An overview of tests for animal tissues in feeds applied in response to public health concerns regarding bovine spongiform encephalopathy. Rev Sci Tech. 22:311-31.

4. Guo Z, Emslie KR. 2002. The Detection of Animal Derived DNA in Stockfeed using Real-Time PCR. Project Number TSE.003. Published by Meat & Livestock Australia Limited.

APPENDIX

TABLE AI. Stockfeed ingredients obtained for Project TSE.003A

Sample	Derived from	Details						Source	ID
MBM		Rendering Conditions	Particle size (mm)	Min Temp (°C)	Pressure (bars)	Time (min)			
1	Ovine	Batch	50	133	3.2	25		Dalriada Meats, Keith	241103
2	Ovine	Batch	<50	133	3	20		Conroy's, Port Pirie	1504B
3	Bovine	Alkaline	1	90	Atmos	90		ADT Rendering P/L	
Gelatin		Tissues used							
1	Bovine	Hide skin						Gelita Australia Pty Ltd, Josephville	32455
Tallow		Rendering conditions				Free Fatty acids (%)	Tallow Grade		
T1	Ovine	Continuous, high temp, min 120°C, centrifugal separation and polished				<2		Fletcher International, Dubbo	FIR TOIA
T2	Bovine	min 120°C					Inedible	Cargill Beef Australia, Wagga Wagga	ETS0291
T3	Bovine	Flow-dry low temp, <0.15% soluble impurities				<1	1-3	Rockdale Beef Pty Ltd, Yanco	RB001
T4	Bovine, ovine	Continuous low temp				2	FAC 11c19	Castricum Bros Pty Ltd, Dandenong	T2
T5	Ovine	Continuous, 40-45 min, 100-140°C, wet						Southern Meats Pty Ltd, Goulburn	SMI
T6	Bovine, porcine	High temp 130-135°C				1-4	Inedible	Northern Co-Op Meat Co, Casino	T1
T7	Bovine, ovine, porcine, chicken	Wet, Preheat 95°C, Press 90°C, Decant 98°C, Separator 90°C				<5		Peerless Holdings P/L, Laverton North	06 329 03
T8	Bovine, ovine, porcine, chicken	Wet, Preheat 95°C, Press 90°C, Decant 98°C, Separator 90°C				10.1	Medium Gut	Peerless Holdings P/L, Laverton North	09 330 03
Milk Powder		Protein content (%)		Fat Content (%)		Type			
M1	Bovine	33.01		7.2		Buttermilk		Murray Goulburn Co-Op Co. Ltd	2987870
M2	Bovine	33.25		0.7		Low Heat Skim		Murray Goulburn Co-Op Co. Ltd	3042469
M3	Bovine	31		12.98		Buttermilk		Murray Goulburn Co-Op Co. Ltd	3030255
M4	Bovine	35		1.25		Medium Heat Skim		Murray Goulburn Co-Op Co. Ltd	3036298
M5	Bovine			26.31		Full-cream		Murray Goulburn Co-Op Co. Ltd	2917719
M6	Bovine			5.27		Buttermilk		Murray Goulburn Co-Op Co. Ltd	2951858
M7	Bovine	35.29				Skim		Murray Goulburn Co-Op Co. Ltd	2934439
M8	Bovine	10.8		1.0		Elite Whey		Murray Goulburn Co-Op Co. Ltd	3130646
M9	Bovine	35.23		3.16		Casein WPC		Murray Goulburn Co-Op Co. Ltd	3138719

TABLE AII. Purity and quantity of DNA extracted from Stockfeed Ingredients

Sample Type	Sample ID	O.D (260nm)	Ratio (260/280)[1]	DNA concentration (ng/uL)[2]
Rendered Ovine MBM				
	241103	0.295	1.742	15.2
	1504B	0.843	1.818	42.3
Gelatin				
	32455	0.008	0.868	0.4
Tallow				
	T1 (FIR T01A)	0.21	1.187	10.1
	T2 (EST 0291)	0.22	1.130	10.3
	T3 (RB 001)	0.132	0.834	6.5
	T4 (T2)	0.148	0.910	7.4
	T5 (SMI)	0.074	0.935	3.5
	T6 (T1)	0.169	0.745	8.4
	T7 (632903)	0.121	0.895	5.5
Milk Powder				
	M1 (2987870)	0.007	0.800	0.4
	M2 (3042469)	0.015	0.988	0.7
	M3 (3030255)	0.007	0.618	0.4
	M4 (3036298)	0.000	0.159	0.0
	M5 (2917719)	0.002	2.754	0.1
	M6 (2951858)	0.003	0.712	0.1
	M7 (2934439)	0.014	0.743	0.7
Spiked stockfeed				
	10% M1 (in PSF RM)	0.393	1.622	19.6
	10% M7 (in PSF RM)	0.376	1.599	18.8
	5% M1 (in PSF RM)	0.358	1.680	17.9
	5% M7 (in PSF RM)	0.352	1.670	17.4
	2% ovine (241103 in PSF RM)	0.430	1.654	21.5
	1% ovine (241103 in PSF RM)	0.425	1.624	21.2
	0.5% ovine (241103 in PSF RM)	0.348	1.670	17.4

The GeneQuant spectrophotometer reads two wave lengths, 260nm and 280nm. The amount of DNA present in a sample of DNA is measured at 260nm wavelength. The amount of contaminating protein in a DNA sample is measured at 280nm wavelength.

[1] The ratio of 260/280nm determines the purity of the DNA sample. A 1.800 ratio indicates very pure DNA. Lower ratios indicate protein contamination.

[2] The DNA concentration is calculated from the absorbance at 260nm in the following manner: 1 O.D. at 260nm for double-stranded DNA = 50 ng/μL of dsDNA, thus $O.D_{260}$ reading x 50 = concentration of DNA in ng/μL

TABLE AIII. PCR screen of MBM, gelatin, milk powder, tallow and stockfeed standards

Sample Type	Sample ID	CT value (average)		Melt Curve Analysis	Result[1]
		40 ng	8 ng		
Controls[2]					
PSF RM (6/01/04)		32.74	34.07		
PSF RM (24/02/04)		32.29	33.78		
0.5% SF RM (6/01/04)		24.03	26.01	Bovine	
0.5% SF RM (24/02/04)		24.33	26.04	Bovine	
Rendered Ovine MBM					
100% Ovine MBM	1504 B		19.51	Ovine	+
100% Ovine MBM	24 11 03	17.05	18.93	Ovine	+
2% Ovine MBM in PSF	24 11 03	26.17	27.42	Ovine	+
2% Ovine MBM in PSF		26.82	28.41	Ovine	+
1% Ovine MBM in PSF		26.70	28.55	Ovine	+
0.5% Ovine MBM in PSF		27.17	28.71		<RT
Alkaline-treated bovine MBM					
100% alkaline-treated bovine MBM		16.31	18.11	Bovine	+
100% Gelatin	32455		33.05	P/dimer	<RT
Milk Powder					
100% Full Cream	M5 (2917719)		20.57	Bovine	+
100% Elite Whey	M8 (3130646)	23.61		Bovine	+
100% Casein WPC	M9 (3138719)	21.69		Bovine	+
100% Buttermilk	M6 (2951858)		19.72	Bovine	+
100% Buttermilk	M3 (3030255)		20.22	Bovine	+
100% Buttermilk	M1 (2987870)		18.76	Bovine	+
10% Buttermilk in PSF	M1 (2987870)	24.01	25.38	Bovine	+
5% Buttermilk in PSF	M1 (2987870)	25.05	25.95	Bovine	+
100% Low Heat Skim milk	M2 (3042469)		22.44	Bovine	+
100% Medium Heat Skim milk	M4 (3036298)		23.94	Bovine	+
100% Skim milk	M7 (2934439)		26.06	Bovine	+
10% Skim milk in PSF	M7 (2934439)	26.44	28.07	Bovine	+
5% Skim milk in PSF	M7 (2934439)	27.84	28.61	Bovine	+
Tallow					
	FIR T01A		45.00		<RT
	EST 0291		45.00		<RT
	RB 001		43.60		<RT
	T2		45.00		<RT
	SMI		44.05		<RT
	T1		37.95	P/dimer	<RT
	632903		36.48	P/dimer	<RT

[1] '+' refers to CT value below the Reporting Threshold hence 'presumptive positive'. '<RT' refers to CT value greater than the CT value at the Reporting Threshold

[2] PSF RM is Plant-based stockfeed Reference Material; 0.5% SF RM is 0.5% (w/w) rendered bovine in plant-based stockfeed Reference Material

TABLE AIV. Factors that may give rise to false positives or negatives in PCR screen

Potential Factor	Risk if not addressed	Potential result	Management of risk
Laboratory sample non-homogeneous since comprised of several sub-samples taken from a heterogeneous lot	The analytical sample and test portion is not representative of the lot	False negative	Method procedure states that laboratory sample is mixed by rolling or homogenisation prior to taking the test portion
Cross-contamination between samples	Incorrect result	False positive	Method procedure includes analysis of suitable positive and negative controls
Non-specific amplification of DNA as primer-dimer or amplification of non-ruminant species due to high homology of genome with primer sequences	Incorrect result	False positive	Method procedure includes melt curve analysis followed by confirmatory analysis using DNA sequencing
PCR inhibitory substances in DNA extract	Incorrect result	False negative	PCR routinely analysed at 2 DNA concentrations and Performance Criteria set to monitor for inhibition

TABLE AV. Summary of tests characteristics

		Neogen Agriscreen for Ruminant Feed	SDI FeedChek™ MBM		NMI PCR Method RD28 screen	Confirmatory Species identification through sequence analysis
Analyte		Ruminant muscle protein	Mammalian, avian or fish connective tissue protein	Mammalian muscle protein	Ruminant cytochrome b gene	
Nominal LOD % (w/w)		1%	0.1%	1%	0.5%	
In-House standards	**Rendering conditions**					
PSF RM		-	-	-	<RT	NA
0.5% SF RM	<EU	+	+	-	+	Bovine
SF B		-	NA	NA	<RT	NA
0.5% ovine MBM in SF B	<EU	+/-	NA	NA	+	NA
SF C		-	NA	NA	<RT	NA
0.5% ovine MBM in SF C	<EU	+	NA	NA	+	NA
SF D		-	NA	NA	<RT	NA
0.5% bovine MBM in SF D	<EU	-	NA	NA	+	NA
SF E		-	NA	NA	<RT	NA
0.5% bovine MBM in SF E	<EU	-	NA	NA	+	NA
4% ovine MBM in PSF RM	EU	+	+	-	+	Ovine
1% ovine MBM in PSF RM	EU	+/-	+	-	+	NA
0.5% ovine MBM in PSF RM	EU	-	+	-	+/-	NA
EU Intercomparison samples						
MAT 1		-	-	-	<RT	NA
MAT 2 - 5% Fishmeal		-	+	-	<RT	NA
MAT 3 - 0.1% PAP 134^{o}C	EU	+/-	+	+/-	<RT	NA
MAT 4 - 0.5% PAP 134^{o}C, 5% Fishmeal	EU	-	+	+/-	<RT	NA
MAT 5 - 0.1% PAP 127^{o}C, 5% Fishmeal	<EU	-	+	-	<RT	NA
MAT 6 - 0.5% PAP 127^{o}C	<EU	+/-	+	+/-	<RT	NA
MAT 7 - 5% Poultry meal		-	+	+/-	<RT	NA
MAT 8 - 0.5% PAP 134^{o}C, 5% poultry meal	EU	+/-	+	+	<RT	NA
Samples from MLA Project TSE.003						

		Neogen Agriscreen for Ruminant Feed	SDI FeedChek™ MBM		NMI PCR Method RD28 screen	Confirmatory Species identification through sequence analysis
Analyte		Ruminant muscle protein	Mammalian, avian or fish connective tissue protein	Mammalian muscle protein	Ruminant cytochrome b gene	
Nominal LOD % (w/w)		1%	0.1%	1%	0.5%	
123-42 Broiler chicken feed 7.75% MBM	<EU	+	NA	NA	+	NA
121-66 Broiler chicken feed 5.75% MBM	<EU	+	NA	NA	+	NA
117-49 Broiler chicken feed 5.5% MBM	<EU	+	+	+	+	NA
127-78 Broiler chicken feed 5.75% MBM	<EU	+	NA	NA	+	NA
127-91 Broiler chicken feed 5.75% MBM	<EU	+/-	+	+	+	NA
590-16 Dairy Cattle feed		-	+	-	<RT	NA
592-14 Dairy Cattle feed		-	+	-	<RT	NA
594-19 Dairy Cattle feed		+/-	NA	NA	<RT	NA
682-77 Dairy Cattle feed		-	NA	NA	<RT	NA
683-03 Dairy Cattle feed		-	NA	NA	<RT	<RT
Commercial samples						
Comm151; Plant-based stockfeed		-	NA	NA	<RT	NA
Comm152; Plant-based stockfeed		-	NA	NA	<RT	NA
Comm9; Stockfeed		-	-	-	+	Bovine
Comm91; Stockfeed		-	+	-	+	Bovine/ovine mix
Comm41; Stockfeed		-	+	-	+	Ovine
Comm121; Fishmeal		-	+	-	<RT	Fish
Comm111; Fishmeal		+	+	+	+	Ovine
Comm112; Fishmeal		+	+	+	+	Ovine
Comm123; Fishmeal		+	NA	NA	+	Ovine
Comm114; Fishmeal		+	NA	NA	+	Bovine
Milk Powder, gelatin, tallow						
100% whey milk powder		NA	-	-	+	NA
100% buttermilk powder		-	-	-	+	NA
5% buttermilk powder		-	-	-	+	NA
100% skim milk powder		-	-	-	+	NA
5% skim milk powder		-	-	-	+/-	NA

	Neogen Agriscreen for Ruminant Feed	SDI FeedChek™ MBM		NMI PCR Method RD28 screen	Confirmatory Species identification through sequence analysis
Analyte	Ruminant muscle protein	Mammalian, avian or fish connective tissue protein	Mammalian muscle protein	Ruminant cytochrome b gene	
Nominal LOD % (w/w)	1%	0.1%	1%	0.5%	
100% gelatin	NR	+	+	<RT	NA
1% gelatin	NA	+	-	NA	NA
100% tallow	-	-	-	<RT	NA

'EU' indicates MBM rendered to EU conditions ie. animal particle size less than 50 mm, autoclaving step at a minimum of 133^{o}C and 3 bars in wet-sterilisation conditions for at least 20 minutes; '<EU' indicates less than the conditions specified by the EU; 'NA' indicates 'Not Analysed'; 'NR' Null Result; '<RT' indicates 'less than the Reporting Threshold'; '-' indicates Not Detected; '+' indicates 'presumptive positive' on screen

TABLE AVI. Factors that may give rise to false positives or negatives in lateral flow strip assays

Potential Factor	Risk if not addressed	Potential result	Management of risk
Strip pads either side of the test strip not removed	Faint bands appear with time	False positive	Method procedure states that for archiving purposes strip pads either side of the test strip must be removed within one hour of test completion
Results not assessed in a timely manner	Faint bands appear with time	False positive	Method procedure states that results from strips must be read within 30 minutes Method procedure includes analysis of negative control with each batch of samples
Sensitivity of test strips drops over time once kit is opened	Positive samples not detected	False negative	Method procedure includes analysis of positive control with each batch of samples
Lateral flow strips are subjective at the LOD, ultimately depending on the analysts' eye sight	Test results vary between analysts, depending on their eye sight/ lighting conditions	False negative or false positive	Results from strips to be assessed by independent analysts to ensure consistency in interpretation
Sample preparation involves boiling and then addition of test strip [1]	Antibody could be denatured if test strip inadvertently placed into boiling sample	False negative	Method procedure involves heating samples in a boiling water bath with no direct contact between the bottom of the sample container and the heating element

[1] This factor is relevant only for the Neogen Agriscreen for Ruminant Feed kit

Abbreviations

AGAL	Australian Government Analytical Laboratories
bp	Base pairs
BSE	Bovine spongiform encephalopathy
C_T	Threshold cycle
DNA	Deoxyribonucleic acid
EU	European Union
LOD	Limit of Detection
MBM	Meat and bone meal
MLA	Meat and Livestock Australia
MMBM	Mammalian meat and bone meal
NMI	National Measurement Institute
nvCJD	Variant Creutzfeldt-Jakob disease
PAP	Processed Animal Protein
PCR	Polymerase chain reaction
PSF RM	Plant-based stockfeed Reference Material
%RSD	Relative Standard Deviation
SDI	Strategic Diagnostics Inc.
$\Delta C_{T_{diln}}$	Difference in C_T values using 8 ng and 40 ng DNA template
$\Delta C_{T_{dupl}}$	Difference in C_T values of duplicate analyses on the same DNA extract