III Detection methods for animal derived proteins

[Methods listed in the Feed Analysis Standards]

1. Sampling, storage and preparation methods for analysis samples

1 Sampling method for analysis samples

Sampling shall be conducted in accordance with the sampling method for microbiological tests [1] and carefully to avoid contamination with substances other than the subject sample.

On sampling, wear plastic gloves, use a sterilized shovel, etc., [2] and sample about 500 g into a sterilized sampling bag.

2 Storage method for analysis samples

Store analysis samples in a refrigerator. When micro-crushed analysis samples are to be stored for a long time (one week or over), store in a freezer (−20°C or less).

3 Preparation method for analysis samples

Meat and bone meal and fish meal are basically not to be crushed. When large particles of bones etc. are contained in such as fish waste, use those that passed a clean mesh sieve (1 mm).

As for a formula feed, crush it until it passes a mesh sieve of 1 mm. [3] During that, wash or exchange the crusher for each sample in order to avoid cross contamination between samples. [4]

2. Identification of bovine derived proteins

2.1 ELISA (I) Note 1[1][2] [Feed Analysis Standards Chapter 17, Section 2, 1.1 (1)]

Scope of application: Feeds of animal origin

A. Reagent preparation

1) Washing solution. Dilute 100 mL of the washing solution (x10 stock solution) Note 2 with water to be 1,000 mL.
2) Substrate ABTS Note 3 solution. Dilute 0.5 mL of the Substrate ABTS stock solution Note 2 with 12 mL of peroxide citrate buffer. Note 2 (Prepare immediately before use. [3])

B. Detection

Extraction. Weigh 5.0 g of an analysis sample, transfer to a 50-mL centrifuge tube, [4] add 15 mL of sodium chloride solution (0.9 w/v%), mix by shaking for 15 minutes to extract, and then centrifuge at 1,000 × *g* for 5 minutes. Transfer 1.5 mL of the supernatant to a microtube (capacity: 1.5 mL), and centrifuge at 5,000 × *g* for 10 minutes to obtain the supernatant to be the sample solution to be subjected to ELISA operation. [5]

In this analysis, conduct parallel analysis for each sample.

ELISA operation. Add 100 μL each of the sample solution, positive control solution, Note 4 negative control solution Note 5 and sodium chloride solution (0.9 w/v%) [6] separately to wells Note 6[7] of the antibody immobilization module, Note 2 [8][9][10], cover the wells with

sealing film, Note 7[11] and then leave at rest at room temperature for 1 hour. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells.

Next, add 25 μL each of biotinylated antibody solution Note 2 to the wells, [12] seal the wells with sealing film, and leave at rest at room temperature for 1 hour. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells.

Then, add 25 μL each of avidine-enzyme complex solution Note 2 to the wells [12], cover the wells with sealing film, and leave at rest at room temperature for 30 minutes. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 μL each of washing solution to the wells.

Add 50 μL each of substrate ABTS solution to the wells [12], and leave at rest protected from light at room temperature for 30 minutes. Moreover, add 50 μL each of the reaction stopping solution Note 2 to the wells [12] to stop color development. Measure absorbance at 405 nm and 492 nm of each well with a microplate reader within 15 minutes, and obtain the measured value as the absorbance at 405 nm minus the absorbance at 492 nm of each well. [13]

Determination. [14] The sample is determined as positive when the mean of measured values of the sample solution is not less than the cutoff, Note 8 and determined as negative when it is less than the cutoff. Conduct the test again if requirements for test validity Note 9 are not met.

Note 1 The method using the ELISA-TEK Cooked Meat Species kit (bovine) (ELISA Technologies) or a method that can produce equivalent results.

2 Use one that is included in the kit.

3 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonate)

4 Use the bovine positive control solution that is included in the kit.

5 Use the three of the porcine and the sheep positive control solutions included in the kit and the chicken positive control solution included in the said Cooked Meat Species kit (poultry) as the negative control solution in this method.

6 Put each solution into more than one well, and determine using the mean of respective measured values.

7 The film shall be applied on the upper side of the antibody immobilization module to prevent evaporation and sublimation of the solutions in the wells.

8 The cutoff is the threshold that is the criterion for determination. The cutoff value here is the mean measured value of the negative control solution multiplied by 2.5.

9 Requirements for test validity are as follows:

- The mean measured value of the positive control solution measured is not less than 8-fold of the mean measured value of the negative control solution.
- The standard deviation value of absorbance of the parallel analysis for each test sample is less than 10% of the mean measured value of the positive control solution.

<<Summary of analysis method>>

This is an analysis method to detect heat-treated bovine derived proteins in a feed by ELISA (sandwich ELISA), which utilizes commercially available "Cooked Meat Speciation Kits" (ELISA Technologies) and is conducted according to the manufacturer's instructions included in the kit.

ELISA (Enzyme Linked Immuno Sorbent Assay) is a method that detects the target protein using the antigen-antibody reaction of protein, and that usually uses cup-shaped containers called wells in which the antibody that specifically reacts with and binds to the target protein as the antigen is applied on the bottom.
This method is a qualitative test and cannot be used as a quantitative test. It does not identify the presence or absence of prion proteins either.

<<Detection sensitivity and specificity>>

- Detection sensitivity
  Bovine derived protein in fish meal: about 1% as bovine meat and bone meal (calculated as the original) Bovine derived protein in meat and bone meal: about 1% as bovine meat and bone meal (calculated as the original)
  Bovine derived protein in chicken meal: about 1% as bovine meat and bone meal (calculated as the original)
  Note that the detection sensitivity may differ by temperature conditions for the heat treatment of a testing sample (material) (manufacturing methods) and the condition of content of ingredients, etc.
- Specificity
  Animal species that is confirmed to be detected: cattle
  Animal species that are confirmed to be undetected: pigs and chickens

<<Notes and precautions>>

[1] Test procedures etc. are according to the “manufacturer’s instructions” included in the kit.
[2] The kit was developed to discriminate meat species in heated processed meat foods, and there is no guarantee that the kit can detect all the target proteins in feed testing.
[3] Mix mildly to the extent not to cause foaming.
[4] Use polypropylene tubes that can be used in a reciprocating shaker.
[5] Oil film or floating oil may be formed at around the upper part of the supernatant. They should be removed as much as possible with a pipette, etc., to prevent contamination into wells.
[6] To reduce difference in time after the addition to the wells as much as possible, it is desirable to dispense the solutions into a spare plate, etc. in advance, and transfer them quickly with a multipipette to the wells.
[7] As well strips may be dropped out during washing, etc., it is preferable to write numbers on well strips with a pencil, etc., in advance.
[8] Experience in pipette operations is needed to prevent variance in reaction.
[9] Avoid touching the inner wall side and the bottom of the well with the tip end during dropping reagents into the well with a pipette. In addition, it is needed to drop a solution directly to the bottom of the well without running on the inner wall. Care should be taken to avoid forming bubbles in a solution and on the solution surface.
[10] After adding the solutions to the wells respectively, mix the solutions in the wells by horizontally and mildly rotating the frame or mildly hitting the corner of the frame to the extent that the solutions are not spattered or spilled.
[11] It is needed to obtain commercially available sealing film separately as it is not included in the kit.
[12] Put the solution in a sample tray, etc., and quickly add to the wells with a multipipette.
[13] Calculate the measured value without blank correction and subject it to determination.
[14] Determination is summarized in Figure 17.2-1.

Sample	→	ELISA		→ ELISA retest		→ Determination
1	↗	1-1	+			Positive
	↘	1-2	+			
2	↗	2-1	-			Negative
	↘	2-2	-			
3	↗	3-1	+	3-3	+	Positive
	↘	3-2	-	3-4	+	
4	↗	4-1	+	4-3	+	Negative
	↘	4-2	-	4-4	-	
5	↗	5-1	+	5-3	-	Negative
	↘	5-2	-	5-4	-	

Figure 17.2-1 Summary of determination by ELISA

2.2 ELISA (II) [Note 1][1] [Feed Analysis Standards Chapter 17, Section 2, 1.1 (2)]

Scope of application: Formula feeds, and feeds of animal origin; however, porcine and chicken derived proteins as well as feeds made from these are excluded. [Note 2]

A. Reagent preparation

1) Extraction solvent. Dissolve 50 mL of the extraction solvent stock solution-I [Note 3] and 2.5 mL of the extraction solvent stock solution-II [Note 3] in water to be 1,000 mL.
2) Washing solution. Dilute 50 mL of the washing solution (x20 stock solution) [Note 3] with water to be 1,000 mL.
3) Standard solutions. Dilute a certain amount of the standard stock solution [Note 3] with the extraction solution accurately to prepare standard solutions of 2-fold, 4-fold, 8-fold and 16-fold dilution.

B. Detection

Extraction. [2] Weigh 4.0 g of analysis sample, [3] transfer to the cup dedicated to a homogenizer, [Note 4] add 36 mL of extraction solvent, and mix with the homogenizer [Note 4] 3 times, for 30 seconds each. Heat this in a boiled water bath for 10 minutes, [4] stand to cool, [5] and then centrifuge at 800-1,000 × *g* for 5 minutes, and filter the supernatant with filter paper (5A). Transfer 1 mL of the filtrate to a microtube (capacity: 1.5 mL), centrifuge at 3,000 × *g* for 10 minutes to obtain the supernatant as the sample solution to be subjected to ELISA operation. [6][7]

ELISA operation. Add 100 µL each of the sample solution, standard solutions, positive control solution [Note 3], negative control solution [Note 3] and extraction solvent (as the blank solution) separately to wells [Note 5][8] [9] [10] of the antibody immobilization module, [Note 3] [11] [12] [13] shake mildly to mix with a module lid [Note 3] closed, and then leave at rest at room temperature for 2 hours. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 µL each of washing solution to the wells. [14]。

Next, add 100 µL each of the enzyme-labeled antibody solution [Note 3] to the wells, [15] shake mildly to mix with a module lid closed, and then leave at rest at room temperature for 30 minutes. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 µL each of washing solution to the wells. [14]

Then, add 100 µL each of the enzyme substrate solution [Note 3] 100 µL to the wells, [15] shake mildly to mix with a module lid closed, and then leave at rest at room temperature

for 10 minutes. Moreover, add 50 μL each of the reaction stopping solution [Note 3] to the wells [15] to stop enzyme reaction. Measure absorbance at 450 nm and 620 nm [Note 6] of each well with a microplate reader within 30 minutes, and obtain the measured value as the absorbance at 450nm minus the absorbance at 620 nm of each well. [16]

Determination. [17] The sample is determined as positive when the mean of measured values of the sample solution is not less than the cutoff, [Note 7] and determined as negative when it is less than the cutoff. Conduct the test again if requirements for test validity [Note 8] are not met.

Note 1 The method using the Morinaga heat-treated bovine protein detection kit (Morinaga Institute of Biological Science, Inc.) or a method that can produce equivalent results.
When the test by this kit is conducted in a feed for which the use or contamination of mammalian derived proteins is prohibited by law, the test result will be positive if the feed contains feed materials of bovine origin which use in the feed are approved, namely milk, milk products, gelatin or collagen. Therefore, care should be taken to use the kit.

2 The test by this method is not applicable to porcine derived proteins (meat and bone meal, hydrolyzed proteins, steamed bone meal, blood meal, plasma protein, etc.) and feeds made from these due to a risk of false positive. These feeds shall be tested by the method in 1.2.

3 Use one that is included in the kit.

4 Use a mill/mixer IFN-700G (Iwatani), laboratory mill/mixer LM-2 (Osaka Chemical) or a product that can produce equivalent results.

5 Put each solution into more than one well, and determine using the mean of respective measured values.

6 It only has to be a single wavelength within the range of 610-650 nm.

7 The mean measured value of the negative control solution multiplied by 2 shall be used as the cutoff.

8 Requirements for test validity are as follows:

- The mean measured value of the positive control solution measured is not less than the mean measured value of the 8-fold dilution of the standard stock solution, and not more than the mean measured value of the 2-fold dilution of the standard stock solution.
- The mean measured value of the negative control solution measured is not more than 0.1 and is less than 2-fold of the measured value of the blank test solution.
- The coefficient of variation of absorbance of the parallel analysis for each test sample is not more than 20%.[18]

<<Summary of analysis method>>

This is an analysis method to detect heat-treated bovine derived proteins in a feed by ELISA (sandwich ELISA), which utilizes commercially available “Morinaga heat-treated bovine protein detection kit” (Morinaga Institute of Biological Science, Inc.) and is conducted according to the manufacturer’s instructions included in the kit.

As for ELISA, see <<Summary of analysis method>> in 2.1.

<<Detection sensitivity and specificity>>

- Detection sensitivity
  Bovine derived protein in formula feeds: about 0.1% as bovine meat and bone meal (calculated as the original)
  Bovine derived protein in fish meal: about 0.1% as bovine meat and bone meal (calculated as the original)
  Bovine derived protein in chicken meal: about 0.1% as bovine meat and bone meal (calculated as the original)
  Note that the detection sensitivity may differ by temperature conditions for the heat treatment of a testing sample (material) (manufacturing methods) and the condition of content of ingredients, etc.
- Specificity
  Animal species that is confirmed to be detected: cattle
  Animal species that is confirmed to be undetected: chickens
  Materials that show false positive due to nonspecific reaction: barley and its processed products, rapeseed and its processed products, malt, malt sprouts, and beer lees.

<<Notes and precautions>>

[1] Test procedures etc. are according to the “manufacturer’s instructions” included in the kit.
[2] Use devices that are washed with a neutral detergent, etc., and then sonicated for about 30 minutes, or soaked in an alkaline washing solution overnight in advance to prevent cross contamination.
[3] Conduct this for a formula feed. The mill should be capable of washing, exchange, and sterilization of sample containers and cutters. Laboratory mill/mixer 300DG (Iwatani) or equivalent is convenient. Crush 200 g or more per sample and mix to be uniform.
[4] Transfer the whole content of the cup to an ice-cooled, screw-capped polypropylene centrifuge tube (V-bottom centrifuge tube from Assist or equivalent), close the cap a little loosely, and heat in a boiling water bath for 10 minutes.
[5] Stand to cool quietly and sufficiently, and conduct the next operation after the sample becomes about room temperature.
[6] Alternatively, after cooling, centrifuge at 3,000 × g for 10 minutes, and then filter with filter paper (5 A) to obtain the filtrate to be the sample solution.
[7] Oil film or floating oil may be formed at around the upper part of the supernatant. They should be removed as much as possible with a pipette, etc., to prevent contamination into wells.
[8] Take out a required number of modules from the bag and set them in a frame. To pack them and set them in order in the frame, set additional blank modules (used and washed modules will do)
[9] As well strips may be dropped out during washing, etc., it is preferable to write numbers on well strips with a pencil, etc., in advance.
[10] To reduce difference in time after the addition to the wells as much as possible, it is desirable to dispense the solutions into a spare plate, etc. in advance, and transfer them quickly with a multipipette to the wells.
[11] Experience in pipette operations is needed to prevent variance in reaction.
[12] Avoid touching the inner wall side and the bottom of the well with the tip end during dropping reagents into the well with a pipette. In addition, it is needed to drop a solution directly to the bottom of the well without running on the inner wall. Care should be taken to avoid forming bubbles in a solution and on the solution surface.
[13] After adding the solutions to the wells respectively, mix the solutions in the wells by

horizontally and mildly rotating the frame or mildly hitting the corner of the frame to the extent that the solutions are not spattered or spilled.

[14] A commercially available automatic plate washer may also be used. As it is needed to conduct this washing sufficiently, the test operator can decide to add the number of washing.

[15] Put the solution in a sample tray, etc., and quickly add to the wells with a multipipette.

[16] Calculate the measured value without blank correction and subject it to determination.

[17] Determination is summarized in Figure 18.2-2.

[18] The coefficient of variation (CV) of absorbance of each test sample measured in repeat was designated as not more than 20% with reference to "Testing method for foods containing allergic substances (PFSB/DFS Notification No. 1106001, Notification from the Director of the Department of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Japan, November 6, 2002)."

3 Identification of ruminant derived proteins

3.1 ELISA [1]

[Feed Analysis Standards Chapter 17, Section 2, 1.2]

Scope of application: Feeds of animal origin (except those derived from fish and shellfish.)

A. Reagent preparation

1) Extraction solvent. Dissolve 14.0 g (1 package) of buffer salt for extraction solvent [Note 2] in water to be 1,000 mL.
2) Washing solution. Dilute 100 mL of the washing solution (x10 stock solution) [Note 2] with water to be 1,000 mL.
3) 1% Positive control solution. [2] Dilute 100 μL of the positive control solution [Note 3] with the extraction solution to be 1 mL.[3]
4) 0.05 % positive control solution. Dilute 50 μL of the 1% positive control solution with the negative control solution [Note 4] to be 1 mL. [3]

B. Detection

Extraction. Weigh 5.0 g of an analysis sample, transfer to a 100-mL stoppered Erlenmeyer flask, add 50 mL of the extraction solvent, mix by shaking for 20 minutes to extract, and then heat this in a boiling water bath for 15 minutes, stand to cool, and then filter with filter paper (5A). [Note 5] Transfer 1 mL of the filtrate to a microtube (capacity: 1.5 mL), and centrifuge at 5,000 × *g* for 10 minutes to obtain the supernatant to be the sample solution to be subjected to ELISA operation. [4]

ELISA operation. Add 100 μL each of the sample solution, 1 % positive control solution, 0.05 % positive control solution, negative control solution and extraction solvent (as the blank solution) separately to wells [Note 6][5] of the antibody immobilization module, [Note 2] [6][7][8], cover the wells with sealing film [Note 7][9] and shake mildly to mix, [10] and then leave at rest at room temperature for 20 minutes. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells. [11]

Next, add 50 μL each of biotinylated antibody solution [Note 2] to the wells, [12] seal the wells with sealing film and shake mildly to mix, and leave at rest at room temperature for 20 minutes. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells. [11]

Then, add 50 μL each of avidine-enzyme complex solution [Note 2] to the wells [12], cover the wells with sealing film and shake mildly to mix, and leave at rest at room

temperature for 20 minutes. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 μL each of washing solution to the wells. [11] Next, add 50 μL each of the substrate TMB Note 8 solution Note 2 to the wells, [12] and leave at rest protected from light at room temperature for 20 minutes. Moreover, add 50 μL each of the reaction stopping solution Note 2 to the wells [12] to stop color development. Measure absorbance at 450 nm of each well with a microplate reader within 15 minutes, and obtain the measured value as the absorbance of each well minus the absorbance of the blank solution.

Determination. [13] The sample is determined as positive when the mean of measured values of the sample solution is not less than the cutoff Note 9 and not less than 2-fold of the measured value of the negative control solution; and determined as negative when it is less than the cutoff or less than 2-fold of the measured value of the negative control solution. Conduct the test again if requirements for test validity Note 10 are not met.

Note 1 The method using the MELISA-TEK RUMINANT KIT for MEAT & BONE MEALS and ANIMAL FEEDS (ELISA Technologies) or a method that can produce equivalent results.

2 Use one that is included in the kit.

3 Use 10 % cattle positive control solution that is included in the kit.

4 Use 10 % porcine positive control solution that is included in the kit.

5 When filtration is difficult due to such as coagulation of oils and fats, centrifuge at 1,000 × *g* for 5 minutes before filtration.

6 Put each solution into more than one well, and determine using the mean of respective measured values.

7 The film shall be applied on the upper side of the antibody immobilization module to prevent evaporation and sublimation of the solutions in the wells.

8 3,3',5,5'-Tetramethylbenzidine

9 The cutoff is the threshold that is the criterion for determination. The cutoff value here is 0.100 as a measured value.

10 Requirements for test validity are as follows:

- The mean measured value of 1% positive control solution is not less than 1.000, the mean measured value of the negative control solution is less than 0.100, and the standard deviation of the measured value of 0.05 % positive control solution is not more than 0.100.

<<Summary of analysis method>>

This is an analysis method to detect heat-treated ruminant derived proteins in a feed by ELISA (sandwich ELISA), which utilizes commercially available "MELISA-TEK RUMINANT KIT for MEAT & BONE MEALS and ANIMAL FEEDS" (ELISA Technologies) and is conducted according to the manufacturer's instructions included in the kit.

As for ELISA, see <<Summary of analysis method>> in 2.1.

<<Detection sensitivity and specificity>>

- Detection sensitivity

  Bovine derived protein in porcine meat and bone meal: about 0.1% as bovine meat and bone meal (calculated as the original)

  Bovine derived protein in chicken meal: about 0.2% as bovine meat and bone meal

(calculated as the original)

Note that the detection sensitivity may differ by temperature conditions for the heat treatment of a testing sample (material) (manufacturing methods) and the condition of content of ingredients, etc.

- Specificity

Animal species that are confirmed to be detected: cattle, water buffaloes, and sheep

Animal species that are confirmed to be undetected: pigs, horses, deer, rabbits, and chickens

The antibody specifically detects muscle among animal tissues, and does not detect milk and blood.

<<Notes and precautions>>

[1] Test procedures etc. are according to the “manufacturer’s instructions” included in the kit.

[2] It is preferable as a simple method for checking the performance of the kit and test instruments to compare the absorbance value with the previous absorbance values using the positive control solution within the measurement range of the microplate reader to be used which has absorbance of not less than 1.000% positive control solution usually gives absorbance near the upper limit of the measurement range of a microplate reader, but may exceed the upper limit of the measurement range of a instrument depending on instrument type. In that case, accurately dilute 1 % positive control solution with the extraction solvent to prepare 0.2 % positive control solution and use it in the test as an additional positive control: it shall be within the measurement range and give an absorbance value not less than 1.000.

[3] Mix mildly to the extent not to cause foaming.

[4] Oil film or floating oil may be formed at around the upper part of the supernatant. They should be removed as much as possible with a pipette, etc., to prevent contamination into wells.

[5] To reduce difference in time after the addition to the wells as much as possible, it is desirable to dispense the solutions into a spare plate, etc. in advance, and transfer them quickly with a multipipette to the wells.

[6] As well strips may be dropped out during washing, etc., it is preferable to write numbers on well strips with a pencil, etc., in advance.

[7] Experience in pipette operations is needed to prevent variance in reaction.

[8] Avoid touching the inner wall side and the bottom of the well with the tip end during dropping reagents into the well with a pipette. In addition, it is needed to drop a solution directly to the bottom of the well without running on the inner wall. Care should be taken to avoid forming bubbles in a solution and on the solution surface.

[9] It is needed to obtain commercially available sealing film separately as it is not included in the kit.

[10] After adding the solutions to the wells respectively, mix the solutions in the wells by horizontally and mildly rotating the frame or mildly hitting the corner of the frame to the extent that the solutions are not spattered or spilled.

[11] A commercially available automatic plate washer may also be used. As it is needed to conduct this washing sufficiently, the test operator can decide to add the number of washing.

[12] Put the solution in a sample tray, etc., and quickly add to the wells with a multipipette.

[13] For the summary of determination, see Figure 17.2-1 in 1.1 <<**Notes and precautions**>> [14] in this section.

4 Identification of porcine derived proteins

4.1 ELISA [Note 1] [Feed Analysis Standards Chapter 17, Section 2, 1.3 (modification of 1.1 (1) Note 1, 4 and 5 in the same section)]

Scope of application: Feeds of animal origin

A. Reagent preparation

1) Washing solution. Dilute 100 mL of the washing solution (x10 stock solution) [Note 2] with water to be 1,000 mL.
2) Substrate ABTS [Note 3] solution. Dilute 0.5 mL of the Substrate ABTS stock solution [Note 2] with 12 mL of peroxide citrate buffer. [Note 2] (Prepare immediately before use.)

B. Detection

Extraction. Weigh 5.0 g of an analysis sample, transfer to a 50-mL centrifuge tube, add 15 mL of sodium chloride solution (0.9 w/v%), mix by shaking for 15 minutes to extract, and then centrifuge at 1,000 × *g* for 5 minutes. Transfer 1.5 mL of the supernatant to a microtube (capacity: 1.5 mL), and centrifuge at 5,000 × *g* for 10 minutes to obtain the supernatant to be the sample solution to be subjected to ELISA operation.

In this analysis, conduct parallel analysis for each sample.

ELISA operation. Add 100 μL each of the sample solution, positive control solution [Note 4], negative control solution [Note 5] and sodium chloride solution (0.9 w/v%) separately to wells [Note 6] of the antibody immobilization module, [Note 2] cover the wells with sealing film, [Note 7] and then leave at rest at room temperature for 1 hour. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells.

Next, add 25 μL each of biotinylated antibody solution [Note 2] to the wells, seal the wells with sealing film, and leave at rest at room temperature for 1 hour. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells.

Then, add 25 μL each of avidine-enzyme complex solution [Note 2] to the wells, cover the wells with sealing film, and leave at rest at room temperature for 30 minutes. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 μL each of washing solution to the wells.

Add 50 μL each of substrate ABTS solution to the wells, and leave at rest protected from light at room temperature for 30 minutes. Moreover, add 50 μL each of the reaction stopping solution [Note 2] to the wells to stop color development. Measure absorbance at 405 nm and 492 nm of each well with a microplate reader within 15 minutes, and obtain the measured value as the absorbance at 405 nm minus the absorbance at 492 nm of each well.

Determination. The sample is determined as positive when the mean of measured values of the sample solution is not less than the cutoff, [Note 8] and determined as negative when it is less than the cutoff. Conduct the test again if requirements for test validity [Note 9] are not met.

Note 1 The method using the ELISA-TEK Cooked Meat Species kit (porcine) (ELISA Technologies) or a method that can produce equivalent results.
2 Use one that is included in the kit.
3 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonate)
4 Use the porcine positive control solution that is included in the kit.
5 Use the bovine and chicken positive control solution that is included in the kit.

6 Put each solution into more than one well, and determine using the mean of respective measured values.
7 The film shall be applied on the upper side of the antibody immobilization module to prevent evaporation and sublimation of the solutions in the wells.
8 The cutoff is the threshold that is the criterion for determination. The cutoff value here is the mean measured value of the negative control solution multiplied by 2.5.
9 Requirements for test validity are as follows:
- The mean measured value of the positive control solution measured is not less than 8-fold of the mean measured value of the negative control solution.
- The standard deviation value of absorbance of the parallel analysis for each test sample is less than 10% of the mean measured value of the positive control solution.

<<Summary of analysis method>>

For the summary of analysis method, see 1.1 <<**Summary of analysis method**>> in this section changing "bovine derived protein" into "porcine derived proteins."

<<Detection sensitivity and specificity>>

- Detection sensitivity
  Porcine derived proteins in fish meal: about 1% as porcine meat and bone meal (calculated as the original)
  Porcine derived proteins in meat and bone meal: about 1% as porcine meat and bone meal (calculated as the original)
  Porcine derived proteins in chicken meal: about 1% as porcine meat and bone meal (calculated as the original)
  Note that the detection sensitivity may differ by temperature conditions for the heat treatment of a testing sample (material) (manufacturing methods) and the condition of content of ingredients, etc.
- Specificity
  Animal species that is confirmed to be detected: pigs
  Animal species that are confirmed to be undetected: cattle, chicken, sheep

<<Notes and precautions>>

See 1.1 <<**Notes and precautions**>> in this section.

5 Identification of poultry derived proteins

5.1 ELISA (II) [Note 1] [Modification of Feed Analysis Standards Chapter 17, Section 2, 2 (1) (Modification of 1.1 (1) Note 1, 4 and 5 in the said section)]

Scope of application: Feeds of animal origin

A. Reagent preparation

1) Washing solution. Dilute 100 mL of the washing solution (x10 stock solution) [Note 2] with water to be 1,000 mL.
2) Substrate ABTS [Note 3] solution. Dilute 0.5 mL of the Substrate ABTS stock solution

[Note 2] with 12 mL of peroxide citrate buffer. [Note 2] (Prepare immediately before use.)

B. Detection

Extraction. Weigh 5.0 g of an analysis sample, transfer to a 50-mL centrifuge tube, add 15 mL of sodium chloride solution (0.9 w/v%), mix by shaking for 15 minutes to extract, and then centrifuge at 1,000 × *g* for 5 minutes. Transfer 1.5 mL of the supernatant to a microtube (capacity: 1.5 mL), and centrifuge at 5,000 × *g* for 10 minutes to obtain the supernatant to be the sample solution to be subjected to ELISA operation.

In this analysis, conduct parallel analysis for each sample.

ELISA operation. Add 100 μL each of the sample solution, positive control solution [Note 4], negative control solution [Note 5] and sodium chloride solution (0.9 w/v%) separately to wells [Note 6] of the antibody immobilization module, [Note 2] cover the wells with sealing film, [Note 7] and then leave at rest at room temperature for 1 hour. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells.

Next, add 25 μL each of biotinylated antibody solution [Note 2] to the wells, seal the wells with sealing film, and leave at rest at room temperature for 1 hour. Remove solutions in respective wells completely, and wash the wells three times in repeat by adding 300 μL each of washing solution to the wells.

Then, add 25 μL each of avidine-enzyme complex solution [Note 2] to the wells, cover the wells with sealing film, and leave at rest at room temperature for 30 minutes. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 μL each of washing solution to the wells.

Add 50 μL each of substrate ABTS solution to the wells, and leave at rest protected from light at room temperature for 30 minutes. Moreover, add 50 μL each of the reaction stopping solution [Note 2] to the wells to stop color development. Measure absorbance at 405 nm and 492 nm of each well with a microplate reader within 15 minutes, and obtain the measured value as the absorbance at 405 nm minus the absorbance at 492 nm of each well.

Determination. The sample is determined as positive when the mean of measured values of the sample solution is not less than the cutoff, [Note 8] and determined as negative when it is less than the cutoff. Conduct the test again if requirements for test validity [Note 9] are not met.

Note 1 The method using the ELISA-TEK Cooked Meat Species kit (chicken) (ELISA Technologies) or a method that can produce equivalent results.

2 Use one that is included in the kit.

3 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonate)

4 Use the chicken positive control solution that is included in the kit.

5 Use the bovine and porcine positive control solution that is included in the kit.

6 Put each solution into more than one well, and determine using the mean of respective measured values.

7 The film shall be applied on the upper side of the antibody immobilization module to prevent evaporation and sublimation of the solutions in the wells.

8 The cutoff is the threshold that is the criterion for determination. The cutoff value here is the mean measured value of the negative control solution multiplied by 2.5.

9 Requirements for test validity are as follows:

- The mean measured value of the positive control solution measured is not less than 8-fold of the mean measured value of the negative control solution.
- The standard deviation value of absorbance of the parallel analysis for each

test sample is less than 10% of the mean measured value of the positive control solution.

<<Summary of analysis method>>

For the summary of analysis method, see 1.1 <<**Summary of analysis method**>> in this section changing "bovine derived protein" into "poultry derived proteins."

<<Detection sensitivity and specificity>>

- Detection sensitivity
  Chicken derived proteins in fish meal: about 1% as chicken meal (calculated as the original)
  Chicken derived proteins in meat and bone meal: about 1% as chicken meal (calculated as the original)
  Note that the detection sensitivity may differ by temperature conditions for the heat treatment of a testing sample (material) (manufacturing methods) and the condition of content of ingredients, etc.
- Specificity
  Animal species that is confirmed to be detected: chickens
  Animal species that are confirmed to be undetected: cattle and pigs

<<Notes and precautions>>

See 1.1 <<**Notes and precautions**>> in this section.

5.2 ELISA (II) [Note 1] [Feed Analysis Standards Chapter 17, Section 2, 2 (2) (Modification of 1.1 (2) Note 1 in the said section)]

Scope of application: Formula feeds, and feeds of animal origin; however, as for feeds of animal origin, porcine derived proteins as well as feeds made from these are excluded. [Note 2]

A. Reagent preparation

1) Extraction solvent. Dissolve 50 mL of the extraction solvent stock solution-I [Note 3] and 2.5 mL of the extraction solvent stock solution-II [Note 3] in water to be 1,000 mL.
2) Washing solution. Dilute 50 mL of the washing solution (x20 stock solution) [Note 3] with water to be 1,000 mL.
3) Standard solutions. Dilute a certain amount of the standard stock solution [Note 3] with the extraction solution accurately to prepare standard solutions of 2-fold, 4-fold, 8-fold and 16-fold dilution.

B. Detection

Extraction. Weigh 4.0 g of analysis sample, transfer to the cup dedicated to a homogenizer, [Note 4] add 36 mL of extraction solvent, and mix with the homogenizer [Note 4] 3 times, for 30 seconds each. Heat this in a boiled water bath for 10 minutes, stand to cool, and then centrifuge at 800-1,000 × *g* for 5 minutes, and filter the supernatant with filter paper (5A). Transfer 1 mL of the filtrate to a microtube (capacity: 1.5 mL), centrifuge at 3,000 × *g* for 10 minutes to obtain the supernatant as the sample solution to be subjected to

ELISA operation.

ELISA operation. Add 100 μL each of the sample solution, standard solutions, positive control solution [Note 3], negative control solution [Note 3] and extraction solvent (as the blank solution) separately to wells [Note 5] of the antibody immobilization module, [Note 3] shake mildly to mix with a module lid [Note 3] closed, and then leave at rest at room temperature for 2 hours. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 μL each of washing solution to the wells.

Next, add 100 μL each of the enzyme-labeled antibody solution [Note 3] to the wells, shake mildly to mix with a module lid closed, and then leave at rest at room temperature for 30 minutes. Remove solutions in respective wells completely, and wash the wells six times in repeat by adding 300 μL each of washing solution to the wells.

Then, add 100 μL each of the enzyme substrate solution [Note 3] to the wells, shake mildly to mix with a module lid closed, and then leave at rest at room temperature for 10 minutes. Moreover, add 50 μL each of the reaction stopping solution [Note 3] to the wells to stop enzyme reaction. Measure absorbance at 450 nm and 620 nm [Note 6] of each well with a microplate reader within 30 minutes, and obtain the measured value as the absorbance at 450nm minus the absorbance at 620 nm of each well.

Determination. The sample is determined as positive when the mean of measured values of the sample solution is not less than the cutoff, [Note 7] and determined as negative when it is less than the cutoff. Conduct the test again if requirements for test validity [Note 8] are not met.

Note 1 The method using the Morinaga heat-treated chicken protein detection kit (Morinaga Institute of Biological Science, Inc.) or a method that can produce equivalent results.

When the test by this method is conducted in a feed for which the use or contamination of poultry derived proteins is prohibited by law, the test result may be positive if the feed contains feed materials of poultry origin which use in the feed are approved, namely eggs and egg products. Therefore, care should be taken to conduct the test by this method.

2 The test by this method is not applicable to porcine derived proteins (meat and bone meal, hydrolyzed proteins, steamed bone meal, blood meal, plasma protein, etc.) and feeds made from these due to a risk of false positive. These feeds shall be tested by the method in 1.2.

3 Use one that is included in the kit.

4 Use a mill/mixer IFN-700G (Iwatani), laboratory mill/mixer LM-2 (Osaka Chemical) or a product that can produce equivalent results.

5 Put each solution into more than one well, and determine using the mean of respective measured values.

6 It only has to be a single wavelength within the range of 610-650 nm.

7 The mean measured value of the negative control solution multiplied by 2 shall be used as the cutoff.

8 Requirements for test validity are as follows:

- The mean measured value of the positive control solution measured is not less than the mean measured value of the 8-fold dilution of the standard stock solution, and not more than the mean measured value of the 2-fold dilution of the standard stock solution.
- The mean measured value of the negative control solution measured is not more than 0.1 and is less than 2-fold of the measured value of the blank test solution.

- The coefficient of variation of absorbance of the parallel analysis for each test sample is not more than 20%.

<<Summary of analysis method>>

For the summary of analysis method, see 1.2 <<**Summary of analysis method**>> in this section changing "bovine derived protein" into "poultry derived proteins."

<<Detection sensitivity and specificity>>

- Detection sensitivity
  Poultry derived proteins in formula feeds: about 0.1% as chicken meal (calculated as the original)
  Poultry derived proteins in fish meal: about 0.1% as chicken meal (calculated as the original)
  Note that the detection sensitivity may differ by temperature conditions for the heat treatment of a testing sample (material) (manufacturing methods) and the condition of content of ingredients, etc.
- Specificity
  Animal species that are confirmed to be detected: chickens, Aigamo duck, and quails
  Animal species that are confirmed to be undetected: cattle and pigs
  Materials that show false positive due to nonspecific reaction: fish egg (confirmed fish species: salmons, codfish, and herring)

<<Notes and precautions>>

See 1.2 <<**Notes and precautions**>> in this section.