II Detection methods for animal derived DNA

[Methods listed in the Feed Analysis Standards]

Section 1, Sampling, storage and preparation methods for analysis samples

1 Sampling method for analysis samples
Sampling shall be conducted 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]

Section 2, Monographs

1 Mammalian derived DNA [Feed Analysis Standards Chapter 16, Section 2]

Use sterilized ultrapure water [5] (purified water that is further purified to be of electric conductivity of 5.9 μS/m or less (resistivity not less than 17 MΩ·cm), hereinafter the same shall apply) as water; use sterilized reagents and devices as appropriate.

1.1 Mammalian derived DNA [Feed Analysis Standards Chapter 16, Section 2, 1.1] Note 1

Scope of application: Feeds except oils and fats

A. Reagent preparation [6]

1) 0.5 mol/L EDTA solution. Dissolve18.6 g of disodium ethylenediaminetetraacetate dehydrate [7] and 2 g of sodium hydroxide in 60 mL of water, adjust the pH with a sodium hydroxide solution (5 mol/L) to 7.9-8.1, [8] and add water to be 100 mL, and autoclave at 121°C for 15 minutes.
2) 1 mol/L Tris-HCl buffer. To 12.1 g of Tris(hydroxymethyl)aminomethane, add 60 mL of water, and further add 4.2 mL of hydrochloric acid to dissolve, adjust the pH with hydrochloric acid to 7.9-8.1, and add water to be 100 mL, and autoclave at 121°C for 15 minutes.

3) Homogenization buffer. [9] Dissolve 1 mL of 1 mol/L Tris-HCl buffer and 8.55 g of sucrose in water to be 100 mL.
4) TE buffer. [10] Dilute 1 mL of 1 mol/L Tris-HCl buffer and 0.2mL of 0.5 mol/L EDTA solution with water to be 100 mL, and autoclave at 121°C for 15 minutes.
5) TAE buffer. Dissolve 4.84 g of Tris(hydroxymethyl)aminomethane, 1.14 mL of acetic acid and 2 mL of 0.5 mol/L EDTA solution 2 mL in water to be 1,000 mL.
6) TBE buffer. Dissolve 10.8 g of Tris(hydroxymethyl)aminomethane, 5.5 g of boric acid and 4 mL of 0.5 mol/L EDTA solution in water to be 1,000 mL.
7) Agarose gel. To 2.5-3.0 g of agarose for electrophoresis, add 100 mL of TAE buffer or TBE buffer, dissolve by heating in a boiling water bath,[11] cool to 50-60°C, pour into a gel mold so that the gel will be 3-4 mm in thickness, insert a comb, and leave at rest horizontally. Remove the comb after the gel is sufficiently solidified.
8) Gel loading buffer. [12] Use the buffer attached to the DNA molecular weight marker Note 2 or prepare as shown below:
Dissolve 25 mg of bromophenol blue, 25 mg of xylene cyanol FF, and 3 g of glycerin in water to be 10 mL, and autoclave at 121°C for 15 minutes.
9) Staining solution. [13] Dissolve 10 mg of ethidium bromide in 1,000 μL of water to prepare the ethidium bromide stock solution, and before use, to 50 μL of the stock solution, add 1,000 mL of TAE buffer or TBE buffer to be the staining solution. [14]
10) Positive control DNA. Use commercially available bovine mitochondrial DNA Note 3 or prepare as shown below:
Extract mitochondrial DNA of cattle (strain: Japanese Black), Note 4 dissolve by the addition of TE buffer to prepare the positive control DNA that contains 10 μg as DNA in 1 mL. [15]
11) Primer solution. [16] Dilute the mammalian detection primer pair Note 5 with water, respectively, to prepare 2 μmol/L 5' primer solution and 3' primer solution.
12) PCR reaction solution. The amount required per PCR tube shall be 4.7 μL of water, 2.0 μL of PCR buffer Note 6, 2.0 μL of 2 mmol/L deoxynucleotide triphosphate mixture solution Note 7, 1.2 μL of 25 mmol/L magnesium chloride solution, 4.0 μL of 2 μmol/L 5' primer solution, 4.0 μL of 2 μmol/L 3' primer solution and 0.1 μL of DNA polymerase solution Note 8 (equivalent to 0.5 unit). Add these reagents to a required number of microtubes (volume: 1.5 mL), respectively, to prepare the PCR reaction solution. [17][18]

B. Detection Note 9

DNA extraction. [6][19] Weigh 100 mg of an analysis sample, transfer to a 2-mL plastic screw-capped tube, add 1.5 g of zirconium dioxide beads Note 10 (1.5 mm in diameter) [20], and further add 1 mL of homogenization buffer, then put the tube in a cell mill Note 11[21], treat at 2,000 rpm for 1 minute, and then leave at rest for 1 minute. Treat this again at 2,000 rpm for 1 minute, cool with ice, and then centrifuge at 4°C, 1,000 × g for 2 minutes. [22] Extract mitochondrial DNA from this supernatant, Note 4[23] dry under vacuum, then dissolve by the addition of 20 μL of TE buffer, [24] and dilute a certain amount of this solution with water to be accurately 10-fold to prepare the DNA sample solution.

Put 18 μL of the PCR reaction solution into a PCR tube (volume: 200 μL), add 2.0 μL of the DNA sample solution, mix by shaking to be the sample solution to be subjected to PCR reaction. [17][25]

At the same time, add 2.0 μL of positive control DNA and 2.0 μL of water respectively to separate PCR tubes which contain 18 μL of the PCR reaction solution in advance and treat similarly to prepare the positive control solution and the negative control solution.

[17][25]

PCR reaction. Put the PCR tubes that contain sample solution, positive control solution and negative control solution to a DNA amplifier and conduct PCR reaction.

Example of PCR reaction conditions

DNA amplifier: Gene Amp System 9700[26] (Applied Biosystems)

Primer: mammalian detection primer pair [anicon5, anicon3] (amplification product size: 176 bp)

Reaction temperature condition: 95°C (9 min retention) →[92°C (30 s retention) →55°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention)

Temperature control condition: 9600 mode [26]

Electrophoresis. Put the agarose gel in a chamber containing TAE buffer or TBE buffer, and pre-run at a constant voltage of 100 V for 10 minutes. Add 1 μL of gel loading buffer to 5 μL each of the sample solution, the positive control solution, the negative control solution and the DNA molecular weight marker Note 2 after completing PCR reaction, mix, and load the whole amount of the solutions separately in the wells of the said agarose gel, and conduct electrophoresis at a constant voltage of 100 V until bromophenol blue migrates to 3-4 cm from the wells. [27]

After completing electrophoresis, put the agarose gel in the staining solution, soak for about 30 minutes, put on a UV transilluminator, illuminate with UV at 365 nm or 312 nm, to identify the presence or absence of PCR-amplified bands. [28]

Determination. The sample is determined as positive when PCR-amplified bands of detection size are detected in the positive control solution, and PCR-amplified bands are not detected in the negative control solution, and PCR-amplified bands that are the same size as the positive control solution are detected in the sample solution.

When PCR-amplified bands are not detected in the positive control solution, or when PCR-amplified bands are detected in the negative control solution, PCR reaction may not have been conducted normally: conduct the test after PCR reaction again.

Also, when the sample is vegetable feed or a formula feed/mixed feed for cattle containing a vegetable feed, conduct the detection of plant derived DNA according to Section 3, 1, and when plant derived DNA is not detected, DNA extraction may have not been conducted normally, therefore conduct DNA extraction again.

Similarly, when the sample is poultry byproduct, conduct chicken derived DNA detection according to Section 3, 2; and when the sample is fish meal etc. (containing fish) or a feed containing fish meal, conduct fish derived DNA detection according to Section 3, 3, respectively, to confirm that DNA extraction is normally conducted.

Note 1 This is a method to detect mammalian derived DNA, and when the test by this method is conducted in a feed for which the use or contamination of mammalian derived proteins is prohibited by law, the test result may be positive if the feed contains feed materials of mammalian origin which use in the feed are approved, namely milk, milk products, gelatin or collagen. Therefore, care should be taken to conduct the test by this method. (See supplementary note).

2 Commercially available 100 bp ladder marker can be used.

3 Bovine mitochondrial DNA positive control (Bex [29])

4 The method using mtDNA Extractor CT kit [30] (Wako Pure Chemicals) or a method that can produce equivalent results.

5 Synthetic DNA for mammalian detection [anicon5, anicon3] (Bex [29]) or a product that can produce equivalent results.

6 10×PCR buffer II (Applied Biosystems), 10×PCR Gold buffer (Applied

Biosystems) or equivalents
7 dNTP Mix (Applied Biosystems) or equivalents
8 Ampli Taq Gold (Applied Biosystems)
9 Cool reagents and devices as appropriate. In addition, conduct test procedures under cool conditions as appropriate, and in a positive-pressure clean bench [17] to avoid contamination.
10 YTZ ball (Nikkato) or equivalents
11 One with revolution of about 2,000 rpm and compatible with 2 mL tubes.

(Supplementary note [31]) When a feed in which milk products such as skim milk and dry whey [32], egg products such as dried whole egg and egg white powder, [33] or gelatin and collagen which use in feeds are approved by law are blended in an amount not more than 10 % [34] or one in which there may be contamination with these [35] is to be tested without their influence for the presence or absence of contamination with animal tissues such as meat and bone meal, conduct treatment according to this section.

Specific gravity separation. [36] Weigh 1.0 g of analysis sample, transfer to a funnel for specific gravity separation that contains chloroform up to 2/3 of its height in advance, [38] stir with a glass bar, [38] and then add chloroform up to the level at 5 mm below the upper end of the funnel for specific gravity separation, and leave at rest for 20 minutes. [39] Discard the supernatant, [40] add chloroform up to 2/3 of the height of the funnel for specific gravity separation, stir, [41] and then add chloroform up to the level at 5 mm below the upper end of the funnel for specific gravity separation, and leave at rest for 10 minutes. Discard the supernatant, and conduct similar procedures.

Hypochlorite treatment. [42] Discard the supernatant in the said funnel for specific gravity separation, and filter the residue with filter paper (5A). [38] Wash the residue on filter paper with a small amount of ethanol, then dry, [43] transfer with a spatula [38] into a 15-mL plastic centrifuge tube, add 10 mL of sodium hypochlorite solution (effective chlorine concentration: 0.5 %), mix by shaking, and leave at rest for 10 minutes. [44]

Enzyme treatment. [45] Aspirate the supernatant in the said centrifuge tube, [46] add 10 mL of ultrapure water to the residue, mix by shaking, and then leave at rest for 5 minutes. Aspirate the supernatant, conduct similar procedures, and then aspirate the supernatant, and add 10 mL of proteinase solution, and leave at rest at 37°C for 18-48 hours. Centrifuge it at 3,500 × *g* for 10 minutes, and aspirate the supernatant. Add 10 mL of ultrapure water to the residue, mix, centrifuge at 3,500 × *g* for 10 minutes, and then aspirate the supernatant, and subject the whole amount of the residue [47] to DNA extraction.

Proteinase solution. Prepare a solution in which 100 mg of proteinase (Proteinase K (Sigma, product number P6556, enzyme activity 41 units/mg) or equivalents) is dissolved in 8 mL of 0.5 mol/L EDTA solution, and add 1 mL of this solution to 49 mL of 0.5 mol/L EDTA solution to prepare the proteinase solution.

<<Summary of analysis method>>

This is a method to detect mammalian derived DNA in a sample by PCR.
The flow sheet concerning PCR in general is shown in Figure 16.2-1.

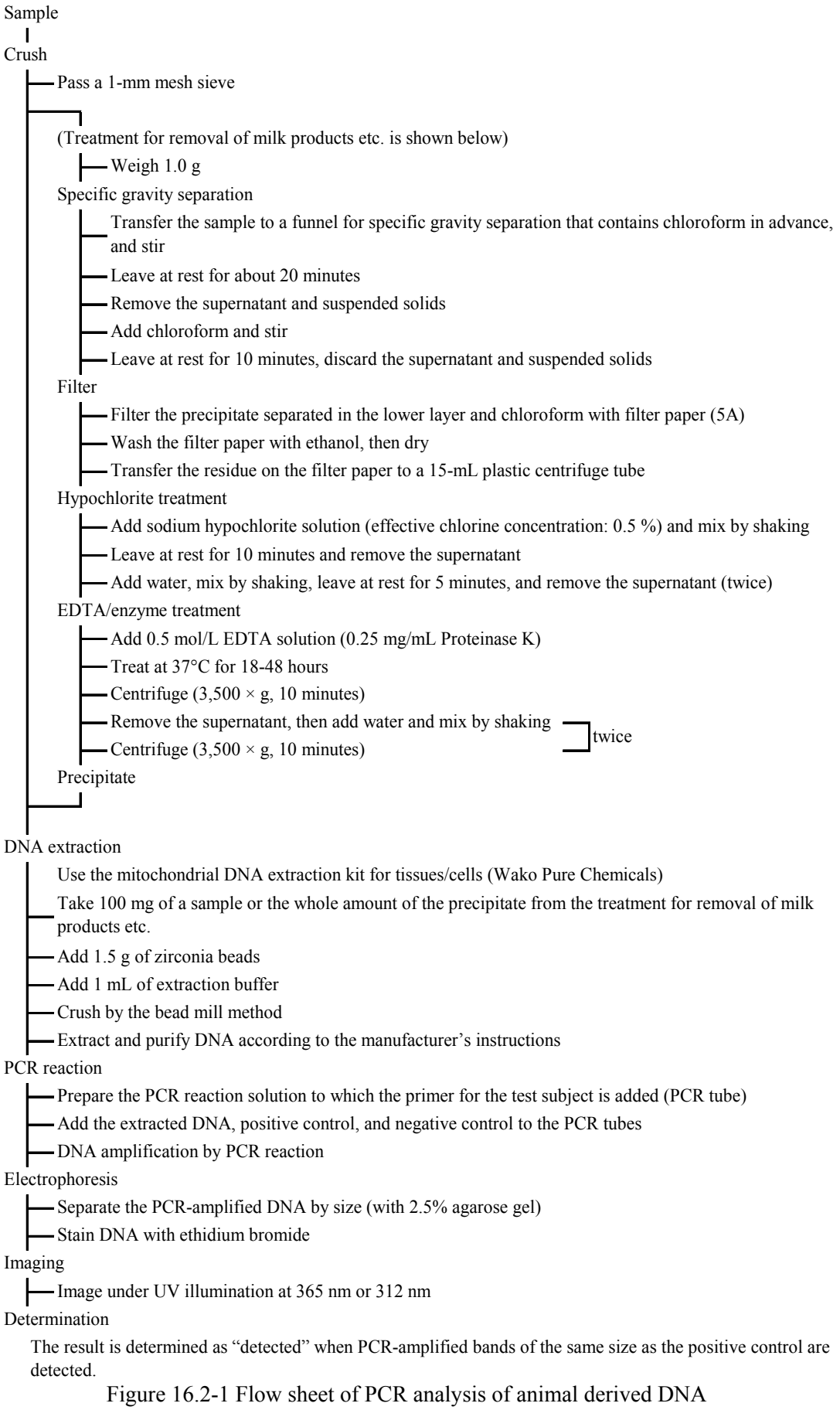

Figure 16.2-1 Flow sheet of PCR analysis of animal derived DNA

<<Precautions in testing>>

1 Precautions in test operations

As PCR has high detection sensitivity and a minute amount of DNA can be detected, it is required to prevent contamination with DNA other than the target DNA with the greatest care during analysis operations. Moreover, as DNA is degraded by DNAse that are present in microorganisms and on the human skin, appropriate operations such as sterilization are needed to avoid contamination with the enzyme. For measures to prevent contamination during PCR test operations and care should also be taken to the items shown below:

1) Use sterilized micropipette tips and exchange them with new ones after each operation. It is desirable to use tips with filter.
2) Wear plastic gloves during analysis operations, and additionally wipe with rubbing alcohol on the glove.
3) Clean the area around the operation site each time by spraying rubbing alcohol and wiping with paper towel etc., and spread plastic wrap etc. to perform test operations on it. Clean all the devices to be used by spraying rubbing alcohol and wiping off.
4) It is desirable to separate the site to conduct sampling and crushing, the site to conduct PCR operations, and the site to conduct electrophoresis.

2 Precautions to ensure test accuracy

1) Conduct DNA extraction for 1 sample in duplicate. It is preferred to conduct PCR reaction in duplicate for each duplicate of the extracted DNA.
2) When PCR reaction is conducted, make sure to react the positive control and the negative control at the same time.
3) For countercheck, it is desirable to use more than one primer. For example, to discriminate animal species such as cattle and sheep, it is preferred to use mammalian detection primer and various animal detection primers.

3 Handling of test results

The result is determined as positive only when both duplicates of extracted DNA produce positive results.

<<Detection sensitivity and specificity>>

Mammalian derived DNA (when mammalian detection primer pair [anicon5, anicon3] is used)

- Detection sensitivity
  Mammalian derived DNA in formula feeds: 0.1-0.01 % as meat and bone meal (content% in the original)
  Note that the detection sensitivity for meat and bone meal may differ by the mixed ratio of the source animal, manufacturing methods (heat treatment conditions) and the content percentage of bone substances.
- Specificity
  Species that are confirmed to be detected: cattle, pigs, sheep, goat, horses, deer, rabbits, and whales
  Species that are confirmed to be undetected: humans, chickens, quails, Alaska pollock, salmons, *Sardinops melanostictus*, *Engraulis japonica*, crabs, shrimps, *Ruditapes philippinarum*, and plants

<<Notes and precautions>>

[2] Exchange gloves and shovels for each sample when a plurality of samples are to be sampled. Autoclave shovels made of stainless steel, etc., wrapped in aluminum foil. It is convenient to have commercially available plastic shovels sterilized by electron beam.

[3] The mill should be capable of washing, exchange, and sterilization of sample containers and cutters. Laboratory mill/mixer LM-1 (Iwatani) or equivalents is convenient.
For formula feeds, crush about 200 g and mix well.

[4] Brush or sonicate, wash well with water, then rinse with distilled water, and dry, and then sterilize if necessary.

[5] Dispense 50-100-mL of ultrapure water in glass reagent bottles (such as medium bottles) and autoclave in advance. Use dedicated ultrapure water as new as possible in a clean bench to prepare the PCR reaction solution.

[6] All the tubes and tips to be used shall be purchased as sterilized or shall be autoclaved. Pack in glass or autoclavable plastic containers by kind, wrap in aluminum foil etc., and autoclave. After autoclaving, dry well at around 70°C for about a few hours.
To prevent contamination, it is preferable to use tips with filter in DNA extraction and the preparation of the PCR reaction solution.

[7] Beware that there are EDTA sodium salts other than disodium salt, such as tetrasodium salt.

[8] Adjust carefully not to exceed pH 8.0. Adjust pH with stirring by a stirrer, making sure that grains of sodium hydroxide are completely dissolved, and then adding 5 mol/L or 1 mol/L sodium hydroxide solution. EDTA·2Na is insoluble in water and is white and turbid at neutral pH, but is dissolved to become clear at around pH 8.0.

[9] “Buffer for Homogenate” included in the mtDNA Extractor CT kit shown in Note 4 can be used.

[10] Commercially available TE buffer (pH 8.0) (Nippon Gene) or equivalents may also be used.

[11] Dissolve using an autoclave or a microwave oven.

[12] One that is attached to DNA size marker at the time of purchase may also be used.

[13] Solution such as commercially available Ethidium Bromide Dropper Bottle (Funakoshi) may also be used. The article contains 0.625 mg/mL as ethidium bromide concentration, and can be used for the preparation of 0.5 μg/mL solution as the final concentration by adding 2 drops (about 80 μL) of the article to 100 mL of electrophoresis buffer.

[14] The ethidium bromide stock solution is 10 mg/mL, resulting in the concentration of 0.5 μg/mL in the electrophoresis buffer.
The ethidium bromide stock solution is photodegradable and should be stored protected from light in a refrigerator. In addition, ethidium bromide is carcinogenic, and make sure to wear gloves to handle, and wear a mask to handle powder. After the end of the test, treat with, for example, filter containing adsorbent (Destaining Bags (Funakoshi) or equivalents), and dispose. Immerse agarose gel stained with ethidium bromide in 5 % sodium hypochlorite solution, and leave under direct sunlight to be photodegraded, and then dispose.

[15] To extract DNA from meat, obtain a lump of meat of known origin, cut off its circumference with such as a clean knife, shred the clean, internal piece of meat, and use about 100 mg of this. To prepare meat micropowder, freeze sliced meat in a freezer or liquid nitrogen, and crush using a cooled crusher. Transfer the crushed meat immediately with a cooled spatula to a plastic container, etc., and store in a freezer.

[16] Primers are available as lyophilized (1 OD) and concentration-adjusted solution (2 μmol/L) from Promega. See the website of Promega

(http://www.promega.co.jp/feedprimer/).

[17] Prepare the PCR reaction solution and the sample solution to be subjected to PCR reaction in a positive-pressure clean bench to prevent contamination with external contaminants. A safety cabinet used in the handling of pathogenic microorganisms is negatively pressurized not to emit pathogens, and is not used for the preparation of the PCR reaction solution, etc.

[18] For preparation operations, place a tube rack on ice, and stand reagents and tubes to be used in this tube rack.
Put 200 μL of 10XPCR Gold buffer, 200 μL of dNTPmix (2 mM) and 120 μL of $MgCl_2$ (25 mM) in a 1.5-mL tube, and mix to prepare buffer mix, dispense approximately by the amount to be used at a time, and store in a freezer in advance. Move the buffer mix, 5′ primer solution and 3′ primer solution (2 μmol/L each) to a refrigerator or room temperature to thaw, and to a 1.5-mL tube, add a required amount of water, buffer mix, primer 5′, primer 3′, and enzyme for PCR reaction in this order to prepare the PCR reaction solution. At that time, mix the amount for (the number of PCR tubes required +1).

[19] In this method, tissues and cells in a sample is crushed by the bead mill method, DNA is extracted using a commercially available mitochondrial DNA extraction kit. Bones contained in fish meal and meat and bone meal as well as plant cell wall can be crushed by the bead mill method.
Formula feeds for cattle are mainly consisted of vegetable feed materials such as maize and soybean meal and contain a large amount of long-chain plant genome DNA. Therefore, extraction efficiency of a minute amount of mitochondrial DNA of animal tissues is poor by total DNA extraction such as the phenol-chloroform method, but high detection sensitivity is produced by this method because mitochondrial DNA can be efficiently extracted by removing long-chain DNA.

[20] It is preferable to dispense by 1.5 g into microtubes after sterilization with an autoclave.

[21] There is lower risk of contamination between samples by using a bead mill because cells can be crushed while each sample is sealed in the tube. In addition, many samples can be treated rapidly by using cell holder that can hold a plurality of samples. On the other hand, common homogenizer treatment can also be used; in that case, prevent contamination between samples by exchanging and washing the container and the mill.

[22] Temperature in the tube will be elevated due to frictional heat by shaking. DNA recovery will be reduced if operation is conducted at elevated temperature, thus cool the cell holder in a freezer in advance and cool the tube by leaving at rest for 1 minute after shaking for 1 minute. If the cell holder of the instrument type cannot be cooled, remove the tube from the cell holder and place on ice for 1 minute to be cooled.

[23] The operation method for the extraction kit is summarized below. For details, see the manufacturer's instruction of the kit. Conduct all the procedures from 1) to 6) quickly under ice-cooled conditions (in an ice box). DNA recovery will be reduced if left at room temperature. From 7) onward, the procedures can be conducted at room temperature.

1) Collect the supernatant to a 1.5-mL microtube, centrifuge at 10,000 × *g* for 10 minutes (4°C), and remove the supernatant.[*1]
2) Add 50 μL of DNA extraction solution I (included in the kit), suspend by pipetting, and further add 100 μL of DNA extraction solution II[*2] (included in the kit), mix, and then cool on ice for 5 minutes.
3) To this, add 75 μL of DNA extraction solution III (included in the kit) cooled on ice in advance, mix with a Vortex mixer, and then cool on ice for 5 minutes.
4) Centrifuge at 12,000 × *g* for 5 minutes (4°C), and then collect about 200 μL of the

supernatant [*3], and transfer to a new 1.5-mL tube.

5) To this, add 300 μL of sodium iodide solution (included in the kit), and further add 500 μL of isopropanol [*4] to mix.
6) Centrifuge at 12,000 × g for 10 minutes [*5] (at room temperature), and then remove the supernatant. [*6]
7) Add 1 mL of 70 % ethanol, mix, and then centrifuge at 12,000 × g for 5 minutes, and remove the supernatant. [*6] Again add 1 mL of 70 % ethanol, mix, and then centrifuge at 12,000×g for 5 minutes, and remove the supernatant [*6] and dry under vacuum.

*1 In the case of high-fat sample, oils and fats will be separated and precipitated in the upper layer or on the inner wall side of the tube; remove them sufficiently with a pipette. If necessary, additionally add about 20 μL of extraction buffer and wash to remove oils and fats etc. adhered on the inner wall side of the tube by pipetting.

*2 Add equal amounts (50 μL each per sample) of DNA extraction solutions IIA and IIB included in the kit to another microtube to mix immediately before use. As IIB is a surfactant, mix slowly with a pipette and avoid foaming. IIB may form precipitate during storage in a refrigerator; examine the presence or absence of precipitate before use, and if there is precipitate, warm with water of about 37°C to dissolve it completely, and return to room temperature to be used.

*3 Care should be taken not to suck the precipitate with a pipette. As this precipitate is liable to be suspended, handle the tube after centrifugation with care, and if the precipitate becomes suspended, centrifuge again to precipitate and then collect the supernatant.

*4 Use JIS Guaranteed Reagent. The solution may become colored due to deterioration of quality over time after opening; dedicate to DNA extraction and use one as new as possible.

*5 After extraction, normally a small amount of DNA precipitate (pellet) can be visually observed. Pellet may not be observed when there is a small amount of DNA, but continue the extraction operation because DNA is adhered to the inner wall side of the tube. Pellet may be colored, such as brown, due to contamination with impurities, but there will be little effect on PCR reaction.

*6 Care should be taken not to suck the pellet with a pipette, and not to touch DNA adhered on the inner wall side of the tube with the end of the tip.

[24] Even if no pellet is observed, dissolve DNA adhered to the tube by repeatedly washing the inner side of the tube by pipetting.

[25] For preparation operations, place a tube rack on ice, and stand reagents and tubes to be used in this tube rack.

[26] The GeneAmp PCR system 9700 has the 9600 mode and the Max mode as temperature elevation speed (Ramp Speed); use the 9600 mode because amplification efficiency may be low in the Max mode.

[27] The dye that migrates ahead during electrophoresis is bromophenol blue.

[28] When a gel image analyzer is used, images can be stored as data.

[29] The original text of the Feed Analysis Standards stipulates as manufactured by Texas Genomics Japan, but the article manufactured by Bex has been supplied by Promega since February 2009. Hereinafter the same shall apply.

[30] Cells contain a large amount of nuclear genome DNA and a minute amount of mitochondrial DNA, etc.; the nuclear genome can be removed and mitochondrial DNA can be extracted in high purity by this kit.

[31] This section is on a pretreatment method for a sample to detect DNA from meat and bone meal (animal bones etc.) in a feed by PCR, which is referred to as treatment for removal of milk products etc. Specific gravity separation with chloroform is used to separate meat and bone meal (animal bones etc.) in a sample from milk products etc., and then by hypochlorite treatment, DNA from cells adhered to animal bones etc. is degraded and removed, and then enzyme treatment is conducted to make bone cell DNA in bone tissues more susceptible to extraction.

[32] As the material milk contains bovine mammary gland cells, bovine mitochondrial DNA is often detected in milk products by PCR. Lactose is also made from whey, and bovine derived DNA may be detected in it depending on the level of purification.

[33] As egg products contain chicken mitochondrial DNA derived from egg cells or egg membrane cells in chicken eggs, it is preferable to conduct the treatment in this section to conduct the detection of poultry derived DNA (2 in this section).

[34] This method is not applicable to feeds in which not less than 10% of milk products are added, such as substitute milk, because validation by collaborative analysis has not been conducted.

[35] These include feeds that are manufactured in the same manufacture line as feeds containing milk products etc., feed additives in which milk products etc. are used as fillers, mixed feeds, and chicken or porcine meat and bone meal (milk products were supplied during breeding). Moreover, the method is also applied to fish meal in which there may be possible contamination with the residue of processed seafood to which egg white, gelatin and meat extract, etc. are added (kamaboko and fish paste).

[36] Conduct in a draft chamber because chloroform is used.

[38] Use sterilized ones and exchange by sample.

[39] The specific gravity of animal bones is 1.9-2.2, which is higher than the specific gravity of chloroform (1.5), and thus animal bones precipitate here. In addition to animal bones, fish bones, calcium carbonate and oyster shells are also precipitated.

[40] Depending on the sample, a layer of suspended solids may be formed in the upper part of chloroform, or it may become suspended and not clarified; remove chloroform and suspended solids sufficiently by decantation.

[41] Mix by pushing the silicon rubber tube connected to the funnel for specific gravity separation with fingers around ten times.

[42] The treatment degrades cells adhered to the surface of animal bone tissues and mammary gland cells that could not be completely removed by specific gravity separation, and degrades and eliminates DNA. DNA from contaminant microbes etc. is also removed.

[43] Air-dry in a clean bench for about 20 minutes.

[44] Care should be taken not to exceed 10 minutes because DNA in animal bone tissues also will become degraded and difficult to be detected. When a plurality of samples are simultaneously treated, care should be taken that the treatment time of each sample does not exceed 10 minutes.

[45] Proteins in animal bone tissues are degraded by enzyme treatment to facilitate the extraction of DNA in bone cells.

[46] Removal can also be performed by pipetting instead of aspiration. Use sterilized pipettes or tips and exchange them by sample. Hereinafter the same shall apply.

[47] If there is a large amount of the residue, sample a part (100 mg) of it.

1.2 Ruminant derived DNA [Feed Analysis Standards Chapter 16, Section 2, 1.2] Note 1

Scope of application: Feeds except oils and fats

A. Reagent preparation [1]

1) 0.5 mol/L EDTA solution. As shown in 1.1 A 1).
2) 1 mol/L Tris-HCl buffer. As shown in 1.1 A 2).
3) Homogenization buffer. As shown in 1.1 A 3).
4) TE buffer. As shown in 1.1 A 4).
5) TAE buffer. As shown in 1.1 A 5).
6) TBE buffer. As shown in 1.1 A 6).
7) Agarose gel. As shown in 1.1 A 7).
8) Gel loading buffer. As shown in 1.1 A 8).
9) Staining solution. As shown in 1.1 A 9).
10) Positive control DNA. As shown in 1.1 A 10).
11) Primer solution. Dilute the ruminant detection primer pair Note 2 with water, respectively, to prepare 2 μmol/L 5' primer solution and 3' primer solution.
12) PCR reaction solution. As shown in 1.1 A 12).

B. Detection Note 3

DNA extraction. [2] As shown in the paragraph 1.1 B DNA extraction.

PCR reaction. [2] As shown in the paragraph 1.1 B PCR reaction; however, primers and reaction temperature conditions in the Example of PCR reaction conditions shall be modified as follows:

Primer: Ruminant detection primer pair [rumicon5D2, rumicon3D5] (amplification product size: 201 bp)

Reaction temperature condition: 95°C (9 min retention) →[92°C (30 s retention) →55°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention)

Electrophoresis [2] As shown in the paragraph 1.1 B Electrophoresis.

Determination. [2] As shown in the paragraph 1.1 B Determination.

Note 1 This is a method to detect ruminant derived DNA, and when the test by this method is conducted in a feed for which the use or contamination of mammalian derived proteins is prohibited by law, the test result may be positive if the feed contains ruminant feed materials which use in the feed are approved, namely milk, milk products, gelatin or collagen. Therefore, care should be taken to conduct the test by this method. (See supplementary note of 1.1).

2 Synthetic DNA for ruminant detection [rumicon5D2, rumicon3D5][3] (Bex) or a product that can produce equivalent results.

3 Cool reagents and devices as appropriate. In addition, conduct test procedures under cool conditions as appropriate, and in a positive-pressure clean bench to avoid contamination.

<<Summary of analysis method>>

This is a method to detect ruminant derived DNA in a sample by PCR.

See 1.1 in this section for the flow sheet concerning PCR in general, notes and precautions, etc.

<<Detection sensitivity and specificity>>

Ruminant derived DNA (when ruminant detection primer pair [rumicon5D2, rumicon3D5] is used)

- Detection sensitivity

Ruminant derived DNA in formula feeds: 0.1-0.01 % as meat and bone meal (content% in the original)

Note that the detection sensitivity for meat and bone meal may differ by the mixed ratio of the source animal, manufacturing methods (heat treatment conditions) and the content percentage of bone substances.

- Specificity

  Species that are confirmed to be detected: cattle, sheep, goat, and deer

  Species that are confirmed to be undetected: pigs , horses, rabbits, rats, mice, humans, whales, chickens, quails, Aigamo duck, Alaska pollock, salmons, *Sardinops melanostictus*, *Engraulis japonica*, flounders, Caranginae, mackerels, *Cololabis saira*, rainbow trouts, *Katsuwonus pelamis*, *Thunnus albacares*, crabs, shrimps, squids, *Ruditapes philippinarum*, and plants

<<Notes and precautions>>

[1] The preparation methods are the same as shown in the paragraphs in this section 1.1 mammalian derived DNA A.

[2] The operation is the same as shown in the same paragraphs in this section 1.1 mammalian derived DNA B.

[3] Primers that were newly developed so that the PCR reaction conditions would be the same as those for the bovine detection primer, etc. for efficient test analysis.

1.3 Bovine derived DNA [Feed Analysis Standards Chapter 16, Section 2, 1.3] Note 1

Scope of application: Feeds except oils and fats

A. Reagent preparation [1]

1) 0.5 mol/L EDTA solution. As shown in 1.1 A 1).
2) 1 mol/L Tris-HCl buffer. As shown in 1.1 A 2).
3) Homogenization buffer. As shown in 1.1 A 3).
4) TE buffer. As shown in 1.1 A 4).
5) TAE buffer. As shown in 1.1 A 5).
6) TBE buffer. As shown in 1.1 A 6).
7) Agarose gel. As shown in 1.1 A 7).
8) Gel loading buffer. As shown in 1.1 A 8).
9) Staining solution. As shown in 1.1 A 9).
10) Positive control DNA. As shown in 1.1 A 10).
11) Primer solution. Dilute the bovine detection primer pair Note 2 with water, respectively, to prepare 2 μmol/L 5' primer solution and 3' primer solution.
12) PCR reaction solution. As shown in 1.1 A 12).

B. Detection Note 3

DNA extraction. [2] As shown in the paragraph 1.1 B DNA extraction.

PCR reaction. [2] As shown in the paragraph 1.1 B PCR reaction; however, primers and reaction temperature conditions in the Example of PCR reaction conditions shall be modified as follows:

Primer: Bovine detection primer pair [cow52, cow31] (amplification product size: 126 bp)

Reaction temperature condition: 95°C (9 min retention) →[92°C (30 s retention) →55°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention)

Electrophoresis [2] As shown in the paragraph 1.1 B Electrophoresis.
Determination. [2] As shown in the paragraph 1.1 B Determination.

Note 1 This is a method to detect bovine derived DNA, and when the test by this method is conducted in a feed for which the use or contamination of mammalian derived proteins is prohibited by law, the test result may 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 conduct the test by this method. (See supplementary note of 1.1).

2 Synthetic DNA for bovine detection [cow52, cow31] (Bex) or a product that can produce equivalent results.

3 Cool reagents and devices as appropriate. In addition, conduct test procedures under cool conditions as appropriate, and in a positive-pressure clean bench to avoid contamination.

<<Summary of analysis method>>

This is a method to detect bovine derived DNA in a sample by PCR.
See 1.1 in this section for the flow sheet concerning PCR in general, notes and precautions, etc.

<<Detection sensitivity and specificity>>

Bovine derived DNA (when bovine detection primer pair [cow52, cow31] is used)

- Detection sensitivity
  Bovine derived DNA in formula feeds: 0.1-0.01 % as meat and bone meal (content% in the original)
  Note that the detection sensitivity for meat and bone meal may differ by the mixed ratio of the source animal, manufacturing methods (heat treatment conditions) and the content percentage of bone substances.
- Specificity
  Species that are confirmed to be detected: cattle
  Species that are confirmed to be undetected: pigs , sheep, goat, horses, deer, rabbits, humans, whales, chickens, Alaska pollock, salmons, *Sardinops melanostictus*, *Engraulis japonica*, crabs, shrimps, *Ruditapes philippinarum*, and plants

<<Notes and precautions>>

[1] The preparation methods are the same as shown in the paragraphs in this section 1.1 mammalian derived DNA A.
[2] The operation is the same as shown in the same paragraphs in this section 1.1 mammalian derived DNA B.

1.4 Porcine derived DNA [Feed Analysis Standards Chapter 16, Section 2, 1.4 and Section 3, 2] Note 1

Scope of application: Feeds except oils and fats

A. Reagent preparation [1]

1) 0.5 mol/L EDTA solution. As shown in 1.1 A 1).

2) 1 mol/L Tris-HCl buffer. As shown in 1.1 A 2).
3) Homogenization buffer. As shown in 1.1 A 3).
4) TE buffer. As shown in 1.1 A 4).
5) TAE buffer. As shown in 1.1 A 5).
6) TBE buffer. As shown in 1.1 A 6).
7) Agarose gel. As shown in 1.1 A 7).
8) Gel loading buffer. As shown in 1.1 A 8).
9) Staining solution. As shown in 1.1 A 9).
10) Positive control DNA. Use commercially available porcine mitochondrial DNA Note 2 or prepare as shown below:
Extract porcine mitochondrial DNA, Note 3 and dissolve by the addition of TE buffer to prepare the positive control DNA that contains 10 μg as DNA in 1 mL.
11) Primer solution. Dilute the porcine detection primer pair Note 4 with water, respectively, to prepare 2 μmol/L 5' primer solution and 3' primer solution.
12) PCR reaction solution. As shown in 1.1 A 12).

B. Detection Note 5

DNA extraction. [2] As shown in the paragraph 1.1 B DNA extraction.

PCR reaction. [2] As shown in the paragraph 1.1 B PCR reaction; however, primers and reaction temperature conditions in the Example of PCR reaction conditions shall be modified as follows:

Primer: Porcine detection primer pair [pig5-6, pig3-6][3] (amplification product size: 83 bp)

Reaction temperature condition: 95°C (9 min retention) →[92°C (30 s retention) →55°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention)

Electrophoresis [2] As shown in the paragraph 1.1 B Electrophoresis.

Determination. [2] As shown in the paragraph 1.1 B Determination.

Note 1 This is a method to detect porcine derived DNA, and when the test by this method is conducted in a feed for which the use or contamination of mammalian derived proteins is prohibited by law, the test result may be positive if the feed contains feed materials of porcine origin which use in the feed are approved, namely gelatin or collagen. Therefore, care should be taken to conduct the test by this method. (See supplementary note of 1.1).

2 Porcine mitochondrial DNA positive control (Bex)

3 The method using mtDNA Extractor CT kit (Wako Pure Chemicals) or a method that can produce equivalent results.

4 Synthetic DNA for porcine detection [pig5-3, pig32-2][3] (Bex), synthetic DNA for porcine detection [pig5-6, pig3-6][4] (Bex) or a product that can produce equivalent results.

5 Cool reagents and devices as appropriate. In addition, conduct test procedures under cool conditions as appropriate, and in a positive-pressure clean bench to avoid contamination.

<<Summary of analysis method>>

This is a method to detect porcine derive DNA in a sample by PCR.

See 1.1 in this section for the flow sheet concerning PCR in general, notes and precautions, etc.

<<Detection sensitivity and specificity>>

Porcine derived DNA (when porcine detection primer pair [pig5-6, pig3-6] is used)

- Detection sensitivity
  Porcine derived DNA in formula feeds: 0.1-0.01 % as porcine meat and bone meal (content% in the original):
  Note that the detection sensitivity for meat and bone meal may differ by the mixed ratio of the source animal, manufacturing methods (heat treatment conditions) and the content percentage of bone substances.
- Specificity
  Species that is confirmed to be detected: pigs
  Species that are confirmed to be undetected: cattle, sheep, goat, horses, deer, rabbits, mice, rats, humans, whales, chickens, quails, Aigamo duck, Alaska pollock, salmons, *Sardinops melanostictus*, *Engraulis japonica*, Caranginae, *Thunnus albacares*, *Katsuwonus pelamis*, *Cololabis saira*, flounders, rainbow trouts, crabs, shrimps, squids, *Ruditapes philippinarum*, and plants.

<<Notes and precautions>>

[1] The preparation methods are the same as shown in the paragraphs in this section 1.1 mammalian derived DNA A.

[2] The operation is the same as shown in the same paragraphs in this section 1.1 mammalian derived DNA B.

[3] Primers that were newly developed so that the PCR reaction conditions would be the same as those for the bovine detection primer, etc. for efficient test analysis.

[4] PCR reaction conditions are: 95°C (9 min retention) →[92°C (30 s retention) →50°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention). The amplification size is 104 bp.

2 Poultry derived DNA [Feed Analysis Standards Chapter 16, Section 2, 2 and Section 3, 3] Note 1

Scope of application: Feeds except oils and fats

Use sterilized ultrapure water as water; use sterilized reagents and devices as appropriate.

A. Reagent preparation [1]

1) 0.5 mol/L EDTA solution. As shown in 1.1 A 1).
2) 1 mol/L Tris-HCl buffer. As shown in 1.1 A 2).
3) Homogenization buffer. As shown in 1.1 A 3).
4) TE buffer. As shown in 1.1 A 4).
5) TAE buffer. As shown in 1.1 A 5).
6) TBE buffer. As shown in 1.1 A 6).
7) Agarose gel. As shown in 1.1 A 7).
8) Gel loading buffer. As shown in 1.1 A 8).
9) Staining solution. As shown in 1.1 A 9).
10) Positive control DNA. Use commercially available chicken mitochondrial DNA Note 2 or prepare as shown below:
    Extract chicken mitochondrial DNA, Note 3 and dissolve by the addition of TE buffer to prepare the positive control DNA that contains 10 μg as DNA in 1 mL.
11) Primer solution. Dilute the chicken detection primer pair Note 4 with water, respectively, to prepare 2 μmol/L 5' primer solution and 3' primer solution.

12) PCR reaction solution. As shown in 1.1 A 12).

B. Detection Note 5

DNA extraction. [2] As shown in the paragraph 1.1 B DNA extraction.

PCR reaction. [2] As shown in the paragraph 1.1 B PCR reaction; however, primers and reaction temperature conditions in the Example of PCR reaction conditions shall be modified as follows:

Primer: Chicken detection primer pair [chick5-1, chick3-1] (amplification product size: 133 bp)

Reaction temperature condition: 95°C (9 min retention) →[92°C (30 s retention) →55°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention)

Electrophoresis [2] As shown in the paragraph 1.1 B Electrophoresis.

Determination. [2] As shown in the paragraph 1.1 B Determination.

Note 1 This is a method to detect poultry derived DNA, and 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 poultry feed materials which use in the feed are approved, namely eggs or egg products. Therefore, care should be taken to conduct the test by this method. (See supplementary note of 1.1).

2 Chicken mitochondrial DNA positive control (Bex)

3 The method using mtDNA Extractor CT kit (Wako Pure Chemicals) or a method that can produce equivalent results.

4 Synthetic DNA for chicken detection [chick5-1, chick3-1] (Bex) or a product that can produce equivalent results.

5 Cool reagents and devices as appropriate. In addition, conduct test procedures under cool conditions as appropriate, and in a positive-pressure clean bench to avoid contamination.

<<Summary of analysis method>>

This is a method to detect poultry derived DNA in a sample by PCR.

See 1.1 in this section for the flow sheet concerning PCR in general, notes and precautions, etc.

<<Detection sensitivity and specificity>>

Poultry derived DNA (when chicken detection primer pair [chick5-1, chick3-1] is used)

- Detection sensitivity
  Poultry derived DNA in formula feeds: 0.1-0.01 % as chicken meal (content% in the original)
  Note that the detection sensitivity for chicken meal may differ by manufacturing methods (heat treatment conditions) and the content percentage of bone substances.
- Specificity
  Species that is confirmed to be detected: chickens and quails
  Species that are confirmed to be undetected: cattle, pigs , sheep, goat, horses, deer, rabbits, humans, whales, Caranginae, *Sardinops melanostictus*, *Engraulis japonica*, *Katsuwonus pelamis*, salmons, mackerels, *Cololabis saira*, Alaska pollock, rainbow trouts, *Thunnus albacares*, crabs, shrimps, *Ruditapes philippinarum*, and plants

<<Notes and precautions>>

[1] The preparation methods are the same as shown in the paragraphs in this section 1.1 mammalian derived DNA A.

[2] The operation is the same as shown in the same paragraphs in this section 1.1 mammalian derived DNA B.

3 Fish derived DNA [Feed Analysis Standards Chapter 16, Section 2, 3 and Section 3, 4] Note 1

Scope of application: Feeds except oils and fats

Use sterilized ultrapure water as water; use sterilized reagents and devices as appropriate.

A. Reagent preparation [1]

1) 0.5 mol/L EDTA solution. As shown in 1.1 A 1).
2) 1 mol/L Tris-HCl buffer. As shown in 1.1 A 2).
3) Homogenization buffer. As shown in 1.1 A 3).
4) TE buffer. As shown in 1.1 A 4).
5) TAE buffer. As shown in 1.1 A 5).
6) TBE buffer. As shown in 1.1 A 6).
7) Agarose gel. As shown in 1.1 A 7).
8) Gel loading buffer. As shown in 1.1 A 8).
9) Staining solution. As shown in 1.1 A 9).
10) Positive control DNA. Use commercially available fish mitochondrial DNA Note 2 or prepare as shown below:
Extract the mitochondrial DNA of *Thunnus albacares*, Note 3 and dissolve by the addition of TE buffer to prepare the positive control DNA that contains 10 μg as DNA in 1 mL.
11) Primer solution. Dilute the fish detection primer pair Note 4 with water, respectively, to prepare 2 μmol/L 5' primer solution and 3' primer solution.
12) PCR reaction solution. As shown in 1.1 A 12).

B. Detection Note 5

DNA extraction. [2] As shown in the paragraph 1.1 B DNA extraction.

PCR reaction. [2] As shown in the paragraph 1.1 B PCR reaction; however, primers and reaction temperature conditions in the Example of PCR reaction conditions shall be modified as follows:

Primer: Fish detection primer pair [FM5, FM3] (amplification product size: 78 bp)

Reaction temperature condition: 95°C (9 min retention) →[92°C (30 s retention) →55°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention)

Electrophoresis [2] As shown in the paragraph 1.1 B Electrophoresis.

Determination. [2] As shown in the paragraph 1.1 B Determination.

Note 1 This is a method to detect fish derived DNA, and the test by this method is conducted in a feed for which the use or contamination of fish derived proteins is prohibited by law, and also conducted as a positive control test for the confirmation of DNA extraction when mammalian or poultry derived DNA in fish meal is tested.

2 Fish mitochondrial DNA positive control (Bex)

3 The method using mtDNA Extractor CT kit (Wako Pure Chemicals) or a

method that can produce equivalent results.
4 Synthetic DNA for fish detection [FM5, FM3] (Bex) or a product that can produce equivalent results.
5 Cool reagents and devices as appropriate. In addition, conduct test procedures under cool conditions as appropriate, and in a positive-pressure clean bench to avoid contamination.

<<Summary of analysis method>>

This is a method to detect fish derived DNA in a sample by PCR.
See 1.1 in this section for the flow sheet concerning PCR in general, notes and precautions, etc.

<<Detection sensitivity and specificity>>

Fish derived DNA (when fish detection primer pair [FM5, FM3] is used)

- Detection sensitivity
  Fish derived DNA in formula feeds: 0.01-0.001 % as fish meal (content% in the original)
  Note that the detection sensitivity for fish meal may differ by the mixed ratio of the source fish, manufacturing methods (heat treatment conditions) and the content percentage of bone substances.
- Specificity
  Species that are confirmed to be detected: *Engraulis japonica*, *Katsuwonus pelamis*, salmons and trouts (*Oncorhynchus keta*, *O. kisutsh*, *O. nerka*, *O. gorbuscha*, *O. masou*, *O. mykiss*, *O. tshawytscha*), mackerels (*Scomber australasicus*, *S. scombrus*, *S. japonicus*) , *Cololabis saira*, perch (*Lateolabrax japonicus, L. maculatus*), sea bream (*Dentex tumifrons*, *Evynnis tumifrons*, *Pagrus major*), codfish (Alaska pollock, Pacific cod), *Sardinops melanostictus*, tuna (*Thunnus orientalis*, *T. albacares*, *T. alalunga*, *T. maccoyii*, *T. obesus*)
  Species that are confirmed to be undetected: eels, Nile perch, crabs (*Paralithodes platypus*, *Lithodes aequispinus*, *Erimacrus isenbeckii*, *Paralithodes camtschaticus*, *Paralithodes brevipes*), shrimps, *Ruditapes philippinarum*, *Cyrenidae*, urchins (purple sea urchin, *Strongylocentrotus intermedius*, *Strongylocentrotus nudus*, *Loxechinus albus*, *Strongylocentrotus droebachiensis*), cattle, pigs, sheep, goat, horses, deer, rabbits, humans, whales, chickens, and plants

<<Notes and precautions>>

[1] The preparation methods are the same as shown in the paragraphs in this section 1.1 mammalian derived DNA A.
[2] The operation is the same as shown in the same paragraphs in this section 1.1 mammalian derived DNA B.

Section 3, Identification of extracted DNA

1 Plant derived DNA [Feed Analysis Standards Chapter 16, Section 3, 1] Note 1

Scope of application: Feeds except oils and fats

Use sterilized ultrapure water as water; use sterilized reagents and devices as appropriate.

A. Reagent preparation [1]

1) 0.5 mol/L EDTA solution. As shown in Section 2, 1.1 A 1).
2) 1 mol/L Tris-HCl buffer. As shown in Section 2, 1.1 A 2).
3) Homogenization buffer. As shown in Section 2, 1.1 A 3).
4) TE buffer. As shown in Section 2, 1.1 A 4).
5) TAE buffer. As shown in Section 2, 1.1 A 5).
6) TBE buffer. As shown in Section 2, 1.1 A 6).
7) Agarose gel. As shown in Section 2, 1.1 A 7).
8) Gel loading buffer. As shown in Section 2, 1.1 A 8).
9) Staining solution. As shown in Section 2, 1.1 A 9).
10) Positive control DNA. Use commercially available maize mitochondrial DNA Note 2 or prepare as shown below:
Extract maize mitochondrial DNA, Note 3 and dissolve by the addition of TE buffer to prepare the positive control DNA that contains about 10 μg as DNA in 1 mL.
11) Primer solution. Dilute plant detection primer pair Note 4 with water, respectively, to prepare 2 μmol/L 5' primer solution and 3' primer solution.
12) PCR reaction solution. As shown in Section 2, 1.1 A 12).

B. Detection Note 5

DNA extraction. [2] As shown in the paragraph 1.1 B DNA extraction.

PCR reaction. [2] As shown in the paragraph 1.1 B PCR reaction; however, primers and reaction temperature conditions in the Example of PCR reaction conditions shall be modified as follows:

Primer: Plant detection primer pair [placon5, placon3] (amplification product size: 140 bp)

Reaction temperature condition: 95°C (9 min retention) →[92°C (30 s retention) →55°C (30 s retention) →72°C (30 s retention)] (45 cycles) →72°C (5 min retention)

Electrophoresis [2] As shown in the paragraph 1.1 B Electrophoresis.

Determination. [2] As shown in the paragraph 1.1 B Determination.

Note 1 This method is conducted as a positive control test for the confirmation of DNA extraction when mammalian, poultry or fish derived DNA in a formula feed/mixed feed for cattle made from vegetable feeds is tested.

2 Maize mitochondrial DNA positive control (Bex)

3 The method using mtDNA Extractor CT kit (Wako Pure Chemicals) or a method that can produce equivalent results.

4 Synthetic DNA for plant detection DNA [placon5, placon3] (Bex) or a product that can produce equivalent results.

5 Cool reagents and devices as appropriate. In addition, conduct test procedures under cool conditions as appropriate, and in a positive-pressure clean bench to avoid contamination.

<<Summary of analysis method>>

This is an analysis method to detect plant derived DNA in a sample by PCR. The method targets the common mitochondrial DNA sequence among maize, cereals and grass that are used in formula feeds for cattle in order to detect them widely. As it is conducted as the positive control test for the confirmation of DNA extraction in the test for DNA from various animals in formula feeds for cattle, it is designed so that PCR reaction conditions etc., will be similar to those of methods for mammalian detection, etc.

See 1.1 in this section for the flow sheet concerning PCR in general, notes and precautions, etc.

<<Detection sensitivity and specificity>>

Plant derived DNA (when plant detection primer pair [placon5, placon3] is used)

- Detection sensitivity
  Plant derived DNA in formula feeds: not determined
- Specificity
  Species that are confirmed to be detected: maize, rice, soybean, sugar beet, wheat, barley, rapeseed, and grass
  Species that are confirmed to be undetected: cattle, pigs, chickens, fish and shellfish, seaweeds, and *Asteraceae*

<<Notes and precautions>>

[1] The preparation methods are the same as shown in the paragraphs in this section 1.1 mammalian derived DNA A.

[2] The operation is the same as shown in the same paragraphs in this section 1.1 mammalian derived DNA B.

2 Porcine derived DNA [Feed Analysis Standards Chapter 16, Section 3, 2]

See Section 2, 1.4 porcine derived DNA in this chapter.

3 Poultry derived DNA [Feed Analysis Standards Chapter 16, Section 3, 3]

See Section 2, 2 poultry derived DNA in this chapter.

4 Fish derived DNA [Feed Analysis Standards Chapter 16, Section 3, 4]

See Section 2, 3 Fish derived DNA in this chapter.