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Method of Test for Animal-Derived Ingredients in Foods- Qualitative Test of Swine Ingredient

1. Scope

This method is applicable to the qualitative test of swine ingredients in foods.

2. Method

The DNA was extracted from the sample, and analyzed by real-time polymerase chain reaction (real-time PCR).

2.1. Working environment: The working platform should be spacious, clean and well-lit. Procedures of sample pretreatment, sample DNA extraction, real-time PCR reagent preparation and assay require separate compartments to avoid cross-contamination. The preparation of real-time PCR reagents should be carried out in a laminar flow hood.

2.2. Equipment (note 1)

2.2.1. Real-time polymerase chain reaction reactor: ABI PRISM 7900HT Sequence Detection System or Roche LightCycler, or equivalent product.

2.2.2. Freeze dryer: temperature ≤ -40°C and vacuum ≤ 133 mBar for sample drying.

2.2.3. Mixer mill: Retsch MM200, or equivalent product.

2.2.4. Vacuum dryer: for DNA drying.

2.2.5. Autoclave.

2.2.6. Laminar flow hood.

2.2.7. Shaker with heat: with temperature control at 55°C and oscillation function.

2.2.8. Micro refrigerated microcentrifuge: centrifugal force ≥ 20,000 × *g*, with temperature control at 4°C.

2.2.9. Microcentrifuge: applicable to various microcentrifuge tubes.

2.2.10. Spectrophotometer: with wavelengths of 260 nm and 280 nm.

2.2.11. Refrigeration equipment: with refrigeration and freezing functions.

2.2.12. Vortex Mixer.

2.2.13. pH meter.

2.2.14. Water bath: capable of controlling water temperature at ± 1.0°C.

2.2.15. Balance: 2000 g ± 0.1 g of maximum weight; 100 g ± 1 mg of maximum weight.

Note 1: The brand of the equipment used or mentioned in this method does not

represent the best of similar equipment; on the contrary, brands of equipment that are not used or mentioned do not represent the poorer of similar equipment.

2.3. Reagent

2.3.1. DNA extraction: ethanol, 96-100%, molecular bioanalytical grade; commercial kits for animal DNA extraction.

2.3.2. Real-time PCR(note 2)

2.3.2.1. Primers and probes for identification test

2.3.2.1.1. Mammals, poultry and fish (target gene: 12S ribosomal RNA as the internal control gene)

Primer F: 12SF, 5'-CAAACTGGGATTAGATACCCCACTA-3'
Primer R: 12SR, 5'-ATCGRTTMTAGAACAGGCTCCTCTAG-3'
Probe P: 12SP, 5'-(FAM)-CACCGCCAAGTCCTTTGRGTTTTARG C-(TAMRA)-3'
PCR amplicon size: 155 bp

2.3.2.1.2. Swine (target gene: 12S ribosomal RNA)

Primer F: P12F, 5'- GGAACAATAGTAAGCACAATCATAGC -3'
Primer R: P12R, 5'- CATAAAAACTTTCGTGTGGTGGA -3'
Probe P: P12P, 5'-(FAM)- CATGTAGAAAATGTAGCCCATTTCT TTCCA-(TAMRA)-3'
PCR amplicon size: 121 bp

Note2: 1. Dilute the synthesized primers and probes with sterile deionized water to an appropriate concentration. After aliquoting, store at -20°C. In addition, keep the probes from light. The 5' end of the probe was labeled with 6-carboxy-fluorescein (FAM), and the 3' end was labeled with 6-carboxytetramethyl-rhodamine (TAMRA).

2. In the sequences of the internal control gene primer and probe, R is the mixed bases (A/G), which contain both A and G, and M is the mixed bases (A/C), which contain both A and C.

2.3.2.2. TaqMan Universal PCR Master Mix: applicable to ABI PRISM 7900HT Sequence Detection System

For real-time PCR, this reagent contains deoxyribonucleotide triphosphate, polymerase, etc. Extra primers, probes and DNA

sample need to be added.

2.3.2.3. LightCycler® FastStart DNA Master HybProbe: applicable to Roche LightCycler

For real-time PCR, this reagent contains deoxyribonucleotide triphosphate, polymerase, 25 mM magnesium chloride solution, etc. Extra primers, probes and DNA sample need to be added.

2.3.3. Reference material: Pork or the reference plasmids No. S201 provided by Taiwan Food and Drug Administration of the Ministry of Health and Welfare was used as a reference material.

2.4. Apparatus and materials(note 3)

2.4.1. Pipette: 10 μL, 20 μL, 100 μL, 200 μL and 1000 μL.

2.4.2. Pipette tip: 10 μL, 20 μL, 100 μL, 200 μL and 1000 μL.

2.4.3. Microcentrifuge tube: 200 μL, 600 μL, 1.5 mL and 2 mL.

2.4.4. Real-time PCR tube: 200 μL.

2.4.5. Real-time PCR glass capillary tube: exclusively for Roche LightCycler.

2.4.6. Glass or plastic bottle: 50 mL, 100 mL, 250 mL, 500 mL, 1000 mL and 2000 mL.

2.4.7. Plastic centrifuge tube: 50 mL.

Note 3: The plastic or glassware used are DNase-free.

2.5. Real-time PCR preparation(note 4)

2.5.1. ABI PRISM 7900HT Sequence Detection System for identification test

5 μM primer F	1.25 μL
5 μM primer R	1.25 μL
3.3 μM probe P	1.7 μL
TaqMan Universal PCR Master Mix	12.5 μL
Sample DNA solution (total 100 ng)	5.0 μL
Sterile deionized water	3.3 μL
Total volume	25.0 μL

2.5.2. Roche LightCycler for identification test

5 μM primer F	1.5 μL
5 μM primer R	1.5 μL
3.3 μM probe P	1.5 μL
LightCycler® FastStart DNA Master HybProbe	2.0 μL
25 mM magnesium chloride	2.4 μL

Sample DNA solution (total 100 ng) .....5.0 μL
Sterile deionized water..... ….6.1 μL
Total volume…….. 20.0 μL

Note 4：Real-time PCR solution should be prepared in an ice bath.

2.6. Sample DNA preparation

2.6.1. Sample pretreatment(note 5)

The dry sample is directly ground into a fine powder with a grinder. After the wet sample is freeze-dried, grind the sample into fine powder with a grinder. The sample must be stored in a dry and frozen environment.

Note 5: 1. Grinding the sample should be carried out in an independent compartment to avoid cross-contamination.
2. The drying time of the wet sample can be adjusted according to the degree of dryness.

2.6.2. DNA extraction

Use commercial kits suitable for animal DNA extraction, and extract the sample DNA according to the operating instruction. Collect the extracted sample DNA solution into a 1.5-mL sterilized centrifuge tube as the sample DNA stock solution. Measure the DNA concentration according to the procedure in section 2.6.3, and store at -20°C.

2.6.3. Measurement of DNA concentration and purity

Take an appropriate amount of the sample DNA stock solution, dilute it with sterile deionized water to an appropriate dilution, and measure the absorbance (O.D.) at wavelengths of 260 nm and 280 nm, respectively. Multiply the absorbance at wavelength of 260 nm by 50 ng/μL and the dilution factor to obtain the concentration of the sample DNA stock solution. The purity of the DNA solution is evaluated by the $O.D._{260}/O.D._{280}$ ratio, and the ratio should be between 1.7 and 2.0.

2.7. Real-time PCR identification test

2.7.1. Real-time PCR operating procedure

2.7.1.1. Real-time PCR－ABI PRISM 7900HT Sequence Detection System

Properly dilute the sample DNA stock solution, primers and probes with sterile deionized water for later use. Take one centrifuge tube, and prepare the real-time PCR solution according to the composition in section 2.5.1. Add the TaqMan® Universal PCR Master Mix, diluted

primers and probes, and sterile deionized water in sequence. Mix well, and aliquot 20 μL into each PCR reaction tube. Add 5 μL of the sample DNA solution, then place the PCR reaction tubes in a centrifuge, and instantaneously centrifuge at 200 × *g*. Transfer them into the real-time PCR reactor, and perform the reaction according to the following conditions. Both positive and negative controls should be prepared in real-time PCR experiments.

Steps	Temperature	Time
1. Incubation	50°C	2 min
2. Initial denaturation (Activation)	95°C	10 min
3. Denaturation	95°C	15 sec
4. Annealing, extension	60°C	1 min
Repeat step 3 and step 4 for 45 cycles		
5. Cooling	35°C	45 sec

2.7.1.2. Real-time PCR－Roche LightCycler

Properly dilute the sample DNA stock solution, primers and probes with sterile deionized water for later use. Take a centrifuge tube, and prepare the real-time PCR solution according to the composition in section 2.5.2. Add the LightCycler® FastStart DNA Master HybProbe, 25 mM magnesium chloride, diluted primers and probes, and sterile deionized water in sequence. Mix well, and then aliquot 15 μL into each glass capillary tube. Add 5 μL of the sample DNA solution, then place the capillary tubes in a centrifuge, and instantaneously centrifuge at 800 × *g*. Transfer them into the real-time PCR reactor, and perform the reaction according to the following conditions. Both positive and negative controls should be prepared in real-time PCR experiments.

Steps	Temperature	Time
1. Initial denaturation (pre-incubation)	95°C	10 min
2. Denaturation	95°C	5 sec
3. Annealing	60°C	25 sec
4. Extension	72°C	8 sec
Repeat step 2 to step 4 for 45 cycles		
5. Cooling	35°C	45 sec

2.7.2. Real-time PCR fluorescence analysis

After the sample DNA is analyzed by the real-time PCR, the results of the reaction can be interpreted by evaluating the fluorescence amplification curve generated by the probe from the screen on the real-time PCR reactor. Both positive and negative controls should be conducted in real-time PCR experiments.

2.7.3. Confirmation

Compare the fluorescence amplification curve of the sample DNA by the real-time PCR with that of the positive control. When the sample DNA and the positive control both show the fluorescence amplification curves generated by the probe, it means that the real-time PCR amplification product of the sample is the target gene fragment of swine, and the sample can be confirmed to contain swine ingredients.

Remark

1. Limit of detection for this test method is 0.1% (on a dry basis).
2. The test results may be affected by the preparation of the DNA, and thus the sample DNA should be assayed for the internal control gene.
3. The reaction parameters can be adjusted depending on the instruments used if the reaction parameters of this method are not applicable.
4. The scope of application of this test method is suitable for foods whose DNA can be extracted, but not for highly processed foods whose DNA has been severely degraded.