Unauthorized genetically modified food detection method

Content

I. Sampling method

1. Flax, wheat, rice, maize, rapeseed sampling

1.1. Grain sampling

Given that the genetically modified foods are unevenly distributed in the samples, a representative sample is collected depending on the sample lot size, packaging and packaging form as followings. During sampling, full consideration must be taken into account so that the grain of the other lots is not mixed, and the disposal or cleaned equipments, containers and packagings, etc. should be used.

Samples are collected from the homogeneous mixture of the grains after thorough mixing, and the grains are ground using the food processor to homogeneous.

For the rapeseed grains, 500 g ground sample (sampling quantity 1 kg) is used. The remaining 500 g is stored as grain. This sample is used for the individual grain testing.

1.1.1. In case of the bag loads

Samples are tested according to following table.

Lot size			No. of unpackaging sample	Sampling mass (kg)	Sampling no.
	≦	15	2	1	1
16	～	25	3	1	1
26	～	90	5	1	1
91	～	150	8	1	1
151	～	280	13	1	1
281	～	500	20	1	1
501	～	1,200	32	1	1
1,201	～	3,200	50	1	1
3,201	～	10,000	80	1	1
10,001	～	35,000	125	1	1
35,001	～	150,000	200	1	1
150,001	～	500,000	315	1	1
	≧	500,001	500	1	1

1.1.2. In case of the bulk

1.1.2.1. At site of the silo loading

Samples are considered as one lot one silo at site of the grain loading, and the samples are collected using the auto-sampler etc. and treat as a sample representative for the entire lot. With an appropriate time interval, the sample aliquots are collected 15 times to have a total of 10 kg ready, and then reduced to a 1 kg or more sample representing each silo.

For those already loaded into the silo, the sampling is performed similarly when transferring to another silo.

1.1.2.2.When transferring into the silo

When transferring into the silo, one silo is defined as one lot. The auto-sampler etc. is used to take samples representing the whole lot. Sample is collected 15 times with an appropriate time interval to make a total of 10 kg ready, and reduce the mass to one sample (1 kg or more) per a silo.

For the loads that have already been transferred into the silo, the sampling is done as described when transferring into another silo.

1.1.2.2. When transferring into the barge

When transferring into the barge, one barge is defined as one lot. The auto-sampler etc. is used to take samples that represents the whole lot. Sample is collected 15 times with an appropriate time interval to make a total of 10 kg ready, and reduce the mass to one sample (1 kg or more) per a barge.

1.1.2.3. Sample collection from the barge

When samples are collected from the barge, one barge is considered as one lot, and five samples are taken from upper, middle and lower layer so that the sample represents the entire lot. More than 10 kg from total of 15 locations are combined and one sample (1 kg or more) is used.

1.1.2.4. Sampling from the container

One container is counted as one lot, and five samples are taken from upper, middle and lower layer so that the sample represents the entire lot. More than 10 kg from total of 15 locations are combined and one sample (1 kg or more) is used.

1.2. Sampling from processed foods

The processed foods are sampled according to the following table. This depends on the lot of interest.

1.2.1. Ground maize processed foods (corn grits, corn flour, corn meal, etc., which are produced from the ground grain)

Sampling is followed according to the directions of 1.1.1. In case of the bag loads.

1.2.2. Other processed foods

Sampling is followed according to the following table.

Size of lot			Unpacking number of samples	Sampling amount (g)	Number of sample
	≦	15	2	120	1
16	～	50	3	120	1
51	～	150	5	120	1
151	～	500	8	120	1
501	～	3,200	13	120	1
3,201	～	35,000	20	120	1
35,001	～	500,000	32	120	1
	≧	500,001	50	120	1

1.3. Sampling from the ground wheat processed foods (wheat powder that is made from the ground wheat grains)

Sampling is followed according to the directions of 1.1.1. In case of the bag loads.

2. Papaya, potato and salmon sampling

2.1. Fresh papaya, fresh potato and raw salmon sampling

Samplings are according to the size of the lot of interest shown in the following table.

Size of lot			Unpacking no. for sampling	No. of sampling
	≦	50	2	2
51	～	500	3	3
501	～	35,000	5	5
	≧	35,001	8	8

2.2. Papaya and potato processed food sampling

Papaya and potato processed foods are sampled according to the size of interest as shown in Table 1.2.2. Sampling amount for the fruit juice and the iced products is set 480 g. In addition, sampling from the processed foods containing low amount of papaya or potato should increase accordingly so that multiple pre-processing of the samples is possible.

Principle

This method is developed to test the fresh and the varieties of the processed foods. Therefore, results may change depending on their characteristics. Here, the principle of the method is indicated to reduce these characteristics.

☑Samples are tested one sample at a time.
☑Samples are at edible part, the part usually used for foods. (For example, sarcocarp without seed or pericarp of the fresh papaya, tuber of potato without skin)
☑Sample of interest is considered not uniformly distributed; therefore, samples should ground and mixed to make homogenous sample.
☑Samples are used at a certain amount regardless of the nature of the samples, such as solid and liquid forms.
☑All steps including sample preparation are done in the lab without air movement and low temperature variations to avoid any possible contaminations.
☑Grinding equipment, containers, weighing instrument, freeze-dried bottle are cleaned using neutral detergent and soaked overnight in an alkaline detergent. Alternatively, the ultrasonic washing machine is used for 30 min. After cleaning, DNA Zap solution ((Thermo Fisher Scientific, Inc., Catalog No. AM9890)) is recommended to degrade the DNA contaminants in the equipment and the containers, and rinse again before use.

*RecheGM200 (Roche), Millser (Iwatani), Force Mil (Osaka Chemicals), Xtreme Blender (Warning), Mortar and pestle can be used.

3. Others

Sampling amount should be enough to the multiple testing for flax, rice and rapeseed.

Ⅱ . Detection methods

Flax (FP967) detection method

In this method, flax grain is tested, and the ion exchange resin type kit method (QIAGEN Inc. Genomic-tip 20/G) is used for the DNA extraction and purification. The DNAs are extracted in duplicate from a sample in parallel, and the qualitative real-time PCR is carried out using the extracted DNA sample solutions.

1. DNA extraction and purification

1.1. The ion exchange resin type DNA extraction and purification kit method (QIAGEN Genomic-tip)

Zero point five grams of the ground samples are placed in a polypropylene centrifuge tube (50 mL capacity), DNAs are extracted and purified by following method using the ion exchange resin type DNA extraction and purification kit (QIAGEN Genomic-tip). In the ground samples, 7.5 mL G2 buffer[*1] and 20 µL α-Amylase[*2] are added and mixed well, and incubate at 37°C for 1 hr. Then, 7.5 mL G2 buffer, 200 µL Proteinase K[*3] and 20 µL RNaseA[*4] are added in the sample, mixed until no samples are remained at the bottom of the tube and incubated at 50°C for 1 hr. During the incubation, the tubes are rocked several times. Then, the samples are centrifuged (5,000×g) at 4°C for 15 min. The resulting 2 mL supernatant is transferred to five 2 mL tubes (total of 10 mL)[*5] and centrifuged (20,000×g) at 4°C for 15 min. The supernatant is loaded on to the QIAGEN Genomic-tip 20/G, which was pre-equilibrated using 1 mL QBT buffer, 1 mL[*5] at a time (total of 5 mL). Then, the column is washed using 2 mL QC buffer[*1] three times, and the column is transferred to a new centrifuge tube, 500 µL QF buffer[*1], which was pre-warmed to 50°C, is loaded and the DNAs are eluted (Eluate 1). The column is transferred to a new centrifuge tube, then the DNAs are further eluted using 500 µL QF buffer[*1] (Eluated 2). An equal volume of isopropanol is added to Eluate 1 and Eluated 2, and each tube is mixed solely for 10 times and the mixture is allowed to stand at room temperature for 5 min. After incubation, the tubes are centrifuged (12,000×g), at 4°C for 15 min and the supernatant is discarded. Five hundred microliters of 70% ethanol is used to rinse the pellet. After centrifugation (12,000×g) at 4°C for 3 min, the pellet is dried. Fifty microliters of 60°C pre-warmed water is added to dissolve the pellet. The DNA solution is added to the next centrifuge tube (Eluate 2) to dissolved the precipitate[*6]. Concentration and purity of the extract DNA sample are estimated using the spectrophotometer.

[*1]G2 buffer, QBT buffer, QC buffer and QF buffer are used from the kit, or all buffers can be prepared according to the manufacture's instructions.

[*2]α-Amylase (high concentration) can be purchased from Nippon Gene Co., Ltd., or other products having equivalent activity can be used.

[*3]Proteinase K (20 mg/mL) can be purchased from Qiagen, or other products having equivalent activity can be used.

[*4]RNaseA (100 mg/mL) can be purchased from Qiagen, or other products having equivalent activity can be used.

[*5]Be careful not to take the precipitate and the film-like upper layer.

[*6]When the precipitate does not dissolve, the solution is incubated at 65°C while mixing for 15 min. If the precipitate does not completely dissolve, the solution is centrifuged (12,000×g) for 3 min at 4°C and the resulting supernatant is transferred to a new centrifuge tube.

1.2. DNA purity measurement in the DNA sample solution and preparation for DNA sample solution stocks

An aliquot of the DNA sample solution is diluted using the sterile distilled water[*1], and the absorbance of the resulting solution is measured at 260 nm and 280 nm (A260 and A280[*2]). The DNA

concentration is measured using A260 (1 A260 equal to 50 ng/μL DNA). If the A260/A280 ratio is 1.7 to 2.0, the DNA is purified[*3] substantially. From the resulting DNA concentration, the DNA sample stocks are prepared by diluting to 50 ng/μL using the sterile distilled water, transferred to the micro-centrifuge tube 15 μL each and stored frozen at <-20°C. The DNA sample stock is used immediately after thawing, the remaining solution is discarded without saving again. If the concentration of the DNA sample stock solution does not meet the concentration defined, the DNA solution is directly used for the PCR sample.

[*1] The degree of the dilution depends since the volume and the concentration range differs among the absorbance measurement instruments.

[*2]A260 is considered the absorbance by the DNA, A280 is considered the absorbance by the protein in the sample.

[*3] No further DNA purification is required when the A260/A280 ratio is outside the range of 1.7-2.0.

2. The qualitative real-time PCR (ABI PRISM™ 7900 or 7500)

FP967 is detected using two real-time PCR methods for FP967 and flax endogenous gene detection. For the FP967 detection, the primers and the probe for detecting the boundary region of the NOS terminator and the spectinomycin resistance gene are used. Also, the primers and the probe for detecting the stearoyl-acyl carrier protein desaturase 2 (SAD) gene sequences are used for the flax positive control. The primers and the probes are dissolved in the sterile distilled water. Sequences of the primers and the probes are as follows.

Primers and probe for detecting FP967
NOST-Spec F: 5'- AGC GCG CAA ACT AGG ATA AA-3'
NOST-Spec R: 5'- ACC TTC CGG CTC GAT GTC TA-3'
NOST-Spec probe: 5'-FAM- CGC GCG CGG TGT CAT CTA TG-BHQ1-3'

Primers and probe for detecting flax endogenous gene
SAD F: 5'- GCT CAA CCC AGT CAC CAC CT -3'
SAD R: 5'- TGC GAG GAG ATC TGG AGG AG -3'
SAD probe: 5'-FAM- TGT TGA GGG AGC GTG TTG AAG GGA-BHQ1-3'

2.1. Preparation for the PCR reaction solution

Total volume of the PCR reaction solution is set 25 μL/well and prepared as follows. After 12.5 μL Universal PCR Master Mix[*1], 0.4 μL 50 μmol/L primer, 0.25 μL 10 μmol/L probe and 22.5 μL sterile distilled water are mixed, 2.5 μL 50 ng/μL DNA sample solution (125 ng) is added. As a negative control PCR reaction, an equal amount of the sterile distilled water is added instead of the DNA sample solution[*2]. The 96-well reaction plate is used and completely sealed[*3] using the sealing applicator. When using the ABI7900HT detection system, place the MicroAmp Optical Cover Compression Pad[*4] above the reaction plate. Each DNA sample solution is tested in duplicate using the FP967 detection and the flax positive control detection methods.

*1 Universal PCR Master Mix

Solution prepared using this reagent has a high viscosity; therefore, the mixing should be carefully

performed. If the mixing is not properly performed, PCR may fail. Universal PCR Master Mix should be mixed and centrifuged before use. Considering that the reaction solution is not easy to be mixed and centrifuged when the Universal PCR Master Mix is contained, it is suggested to pipette all the solution at the bottom of the plate.

*2 Non-Template Control (NTC)

Upon addition of the DNA sample solution, the NTC is prepared by adding 2.5 μL water in place of the DNA sample solution.

*3 The 96-well plate, the adhesive film and the adhesive film applicator

Use the MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.). When sealing the reaction film, refer to the manufacture's instruction.

*4 Use the MicroAmp Optical Cover Compression Pad in case of the ABI PRISMTM 7900, not in case of the ABI PRISM™ 7500.

2.2. Setting plate information

To start reaction, the plate information (sample position, types and probe characteristics) must be set. The sample ("NTC": Non-Template Control, "UNKN": DNA sample solution) are set according to the positions of the samples prepared. Also with respect to the probe characteristics, use "FAM" as a reporter and "Non Fluorescent" as a quencher for both NOST-Spec and SAD. In addition, the passive reference is set to the "ROX". The run mode is set to the 9600 emulation mode.

2.3. PCR amplification

The reaction plate is set to the instrument, then the reaction is started. The reaction condition is as follows. After pre-heating at 50°C for 2 minutes and 95°C at 10 minutes, the thermal cycles are started as follows: 45 cycles of 95°C for 15 sec and 60°C for 1 min. After making sure that the remaining time is zero when the reaction is terminated, and the analysis of the measurement is performed.

3. Real-time PCR data analysis and result report (**see Figure 1**)

In both FP967 and endogenous detection method, the baseline of the real-time PCR amplification curve is set to cycles 3–15. The normalized reporter signal (ΔRn) threshold for plotting cycle threshold (Ct) values is set to 0.2 during the exponential amplification of the fluorescence (FAM) in the multicomponent plots.

The results are judged using all four test wells (duplicate test per a sample).

In the DNA sample solution,

(1) In a duplicate test, if the Ct value <43 in two parallel wells for the endogenous positive control test is obtained and the Ct value <43 in all wells for FP967 detection method are obtained, the sample is contaminated with FP967.

(2) In a duplicate test, if the Ct value <43 in two parallel wells for the endogenous positive control test is obtained and Ct value <43 in all wells for the FP967 detection method are not obtained, the sample is not contaminated with FP967.

(3) In FP967 detection method, if result of all wells did not match, the second DNA extraction and purification from the sample after grinding and homogenization are performed. Then, the qualitative

real-time PCR method is performed. If the FP967 positive is not obtained, the sample is decided not contaminated with FP967.

In a duplicate DNA samples prepared, the endogenous positive control test (total of 4 wells) is considered positive when the Ct values <43 in two parallel wells are obtained. When the Ct values <43 in all wells for FP967 detection method are obtained, the sample is consider contaminated with FP967.

Note that the multicomponent analyses is required in the case of FP967 positive---making sure that the exponential increase in the fluorescence intensity of the FAM is observed visually, and that the definite decrease of the ROX fluorescent intensity and the gradual increase of the FAM fluorescence intensity are observed.

In addition, if the Ct value <43 in both two wells in parallel for the positive control tests cannot be obtained, the DNA sample solution is prepared again according to "1. DNA extraction and purification" from the sample. If the Ct value <43 in both parallel wells is not obtained, the sample is considered not testable.

Wheat (MON71200, MON71700, MON71800) detection method

This method is intended to test wheat grain or ground wheat processed products. DNAs were extracted and purify from each sample in duplicate, and the obtained DNA sample was used as a template for qualitative real-time PCR testing using two wells in parallel.

1. DNA extraction and purification

1.1. Cleaning and grinding of the sample (in the case of grain)

The wheat grain is washed using three times the volume (weight per volume) of 1% SDS aqueous solution, rinsed using water and dried for 40 minutes at 40ºC using the oven. When dried, the sample is ground using a food processor.

1.2. Silica gel membrane type kit method (DNeasy Plant Maxi Kit, QIAGEN) *1

The sample (1 g) is weighed into a 50 mL-volume tube, and 10 µL 100 mg/mL RNase A*2 and 5 mL AP1 buffer*3are added and mixed vigorously using a vortex mixer. Sample was incubated at 65ºC for 1 hour, with mixing by inverting the tube two or three times. After the addition of 1.8 mL P3 buffer*4, mix using a vortex mixer, and stand for 15 minutes in ice water. Using swing-type centrifuge, the sample is centrifuged for 15 minutes at room temperature at the speed of 3,000×g. Four point five milliliters of the supernatant were collected, loaded onto a QIAshredder Maxi spin column. The sample is subjected to a swing-type centrifugal separator (3,000 × g, room temperature, 5 minutes). Four milliliters of the supernatant were collected and transferred to a new 50 mL-volume tube. Six mililitres of AW1 buffer*5 is added and mixed vigorously using a vortex mixer. The whole solution is loaded onto the DNeasy Maxi spin column, subjected to a swing-type centrifugal separator (3,000×g, room temperature, 5 minutes). Discard the flowthrough, 12 mL AW2 buffer*6 is added into the column, subjected to a swing-type centrifugal separator (3,000 × g, room temperature, 15 minutes). The column was transferred to a new 50 mL-volume tube, added 1 mL sterile water that had been pre-warmed to 65ºC into the column. After standing for 5 minutes at room temperature, the sample is subjected to a swing-type centrifuge (3,000×g at room temperature for 10 minutes). Two mililitres of eluate is transferred into sample tube, added an equal volume of isopropanol. After gently mixing10 times, the sample is allowed to stand at room temperature for 5 minutes. After centrifugation at 12,000 × g at 4ºC for 15 minutes, the supernatant is discarded. Five hundred microliters of 70% ethanol are added to rinse the pelette. After centrifugation at 12,000×g at 4ºC for 3 minutes, the supernatant is completely discarded*7, and the precipitate is dried. The precipitate is dissolved in 130 µL water, and used as the DNA sample stock solution.

* 1 Throughout the experiment, make sure to avoid from contaminating.
* 2 Use the one in the kit or separately purchase from QIAGEN (Cat. No. 19101), or use those that have equivalent peformance.
* 3 Use the one in the kit or separately purchase from QIAGEN (Cat. No. 1014630) is used.
* 4 Use the one in the kit or separately purchase from QIAGEN (Cat. No. 19053) is used.
* 5 Use the one in the kit or separately purchase from QIAGEN (Cat. No. 19081) is used.
* 6 k Use the one in the kit or separately purchase from QIAGEN (Cat. No. 19072) is used.
* 7 If the precipitate is not visible, supernatant should be carefully removed by avoiding touching the bottom of the tube.

1.3. Preparation and storage of DNA sample

Follow as previously described.

2. Qualitative real-time PCR detection method

To detect GM wheat, real-time PCR methods using primers and probe set for MON71200, MON71700 or MON71800 detection and wheat positive control test are used. Primers and probes were designed at the boundary region specific to the wheat genomic sequences and transgenic vector sequences. Also, proline-rich protein (PRP) gene detection method is used for wheat positive control test. Primers and probes were dissolved in water and separately stored. Sequences of the primers and probes are as follows.

Primers and probes for MON71200 detection test

MON71200-3' junction-1F: 5'-CAC GAC GGT CAT CGA GC-3'

MON71200-3' junction-1R: 5'-CCG TTC GTC ATT GAC TGT T-3'

MON71200-3' junction-P*: 5'-HEX-CAT ACG GAA/ZEN/AAG ATG CTG CAG GGA ATA TAT TGA AC-IABkFQ-3'

* MON71200-3' junction-P is a double-quencher probe using ZEN and IowaBlack™(IABkFQ) that can be ordered at HPLC-purified grade to Integrated DNA Technologies (IDT).

Primers and probes for MON71700 detection test

71700 forward primer: 5'-CCA TCA TAC TCA TTG CTG ATC CAT GT-3'

71700 reverse primer: 5'-CGG CAT GCG CCA ATC AGT-3'

71700 FAM-probe: 5'-FAM-TTC CCG GAC AGC GGC GG-TAMRA-3'

Primers and probes for MON71800 detection test

SQ0718: 5'-TTC TTC TCT CTC TTT GAA TCT CAA TAC AA-3 '

SQ0719: 5'-CCC CCA TTT GGA CGT GAA-3 '

PB0101: 5'-FAM-TCC CCC TCT CTA ATTC-MGB-3 '

Primers and probe for the wheat positive control test

PRP8F: 5'-GCA CCC ATG AGT ACT ATT CTG TA-3 '

PRPds6R: 5'-TGC AAA CGA ATA AAA GCA TGTG-3 '

PRP-Taq5: 5'-FAM-CTG TGC ACA TGA CTC AGT TGT TCT TTC GTG-TAMRA-3 '

2.1. Preparation of the real-time PCR reaction solution[*1]

PCR reaction solution is prepared in 25 μL/well as follows.

For wheat positive control test, MON71700 detection test and MON71800 detection test, the reactions were prepared as follows.

Twelve point five microliters of FastStart Universal Probe Master (Rox) [*2] (Roche Diagnostics), 0.25 μL each primer solution (50 μmol/L each), 0.5 μL the target probe solution (10 μmol/L) were mixed, and adjusted total volume to 20 μL using water, then 5 μL (10 ng/μL) DNA sample solution is added[*3].

For MON71200 detection test, the reactions were prepared as follows.

Twelve point five microliters of FastStart Universal Probe Master (Rox) [*2] (Roche Diagnostics), 0.2 μL MON71200-3' junction-1F primer (50 μmol/L), 0.4 μL MON71200-3' junction-1R primer (50

μmol/L), 0.5 μL the target probe solution (10 μmol/L) were mixed, and adjusted total volume to 20 μL using water, then 5 μL (10 ng/μL) DNA sample solution is added[*3].

As a blank PCR reaction solution[*4], "without DNA sample solution" is also prepared.

After preparation, the plate is completely sealed, making sure to avoid seal wrinkles and any air bubbles inside the reaction by tapping on the plate corner.

[*1] Genuine 96-well plate and seal recommended for each real-time PCR system are used.
[*2] Instead of using FastStart Universal Probe Master (Rox) (Roche Diagnostics), any master mix having equivalent performance can be used. Be careful when mixing and transferring the sample solution containing the master mix due to its high viscosity.
[*3] Each reagent is thawed at room temperature and keep on ice until use.
[*4] Non-Template Control (NTC) is set to use water instead of DNA sample solution and prepared using 5 μL added per a well.

2.2. Setting the plate information

Be sure the position of samples and probe characteristics, the real-time PCR system is setup according to the instrument's manual. As the probe characteristics, "FAM" as the Reporter and "Non Fluorescent" as the quencher for MON71200, "FAM" as the Reporter and "TAMRA" as the quencher for MON71700, "FAM" as the Reporter and "Non Fluorescent" as the quencher for MON71800, "FAM" as the Reporter and the Quencher "TAMRA" in wheat positive control test. In addition, Passive Reference is set to "ROX". Sample Volume is set to 25 μL.

2.3. PCR amplification

The reaction condition is as follows. After holding 2 minutes at 50ºC and 95ºC for 10 minutes, the amplification reaction of 48 cycles is run by thermal cycle of 95ºC for 15 seconds, 60ºC for 1 minute per a cycle.

3. Result analysis (**see Figure 1**)

The results should be confirmed by exponential amplification curve and fluorescence intensity (FAM or HEX) change. If an exponential amplification curve on the Amplification plot is confirmed using GM wheat detection method, GM wheat-positive is suspected.

First, the following scheme is followed to analyze the results.

In each DNA sample solution,
(1) Ct values of less than 43 in a duplicate well in parallel for wheat positive control test and in a duplicate well in parallel for GM wheat detection test have been obtained, the sample is considered positive.
(2) Ct values of less than 43 in a duplicate well in parallel for wheat positive control test and not in a duplicate well in parallel for GM wheat detection test have been obtained, the sample is considered negative.
(3) Ct values of less than 43 in a duplicate well in parallel for wheat positive control test and the results from duplicate wells in parallel for GM wheat detection test were not equivalent, “1. DNA

extraction and purification" should be repeated and re-tested. If the results from a duplicate test in parallel is not equivalent, the sample should be GM wheat-negative.

If the Ct value of at least less than 43 in one well can not be obtained from wheat positive control test using DNA sample obtained from the second trial, then the sample is considered impossible to analyze.

Subsequently, the results were determined if GM wheat is contaminated or not based of the above results. If DNA samples from duplicated preparations are all positive, the samples is considered positive (Table 1 below). When either of the prepared sample is negative, the sample is considered free of GM wheat.

Table 1. The results consider to contain GM wheat

	wheat positive control test	GM wheat detection test		
		MON71200	MON71700	MON71800
DNA sample (1)	Positive	Positive	Positive	Positive
DNA sample (2)	Positive	Positive	Positive	Positive
		↓	↓	↓
		MON71200 contaminated	MON71700 contaminated	MON71800 contaminated

Rice (63Bt, NNBt, CpTI) detection method

In this method, rice and rice products (only those processed products that contain rice as a major ingredient and are unheated or through low degree of processing, for examples, rice flour, rice vermicelli, etc.) are subjected for testing. For DNA extraction and purification, the ion exchange resin type kit method (QIAGEN Inc. Genomic-tip 100 /G) or the silica gel membrane type method (NIPPON GENE GM quicker 2) are used. DNA is extracted duplicate from a sample in parallel, and the qualitative real-time PCR is subsequently carried out using the DNA sample solutions.

1. DNA extraction and purification

1.1. The ion exchange resin type DNA extraction and purification kit method (QIAGEN Genomic-tip 100/G)

If the DNA is prepared more than enough, the amount of buffers and enzymes required for the DNA extraction and purification can be reduced in half.

The homogenously ground 2 g sample is weighed in a polypropylene centrifuge tube (50 mL capacity), and 15 mL G2 buffer*[1] is added. The sample solution is mixed. Into the homogenized samples, 12 µL α-Amylase[*2] and 60 µL RNaseA[*3] are added and incubated at 37°C for 30 min. During the incubation, the tubes are mixed several times. Then, 60 µL Proteinase K[*4] is added and mixed well, then incubated at 65°C for 30 min. During the incubation, the tubes are mixed several times. Then, the samples are centrifuged (3,000×g) at 4°C for 15 min. The resulting supernatant is transferred onto the QIAGEN Genomic-tip 100/G, which was pre-equilibrated using 4 mL QBT buffer*[1]. Then, the column is washed using 7.5 mL QC buffer[*1] three times and transferred to a new centrifuge tube, then 2 mL QF buffer[*1], which was pre-warmed to 50°C, is loaded to elute the DNA. An equal volume of isopropanol as the DNA eluent is added and mixed solely. Then, transfer the DNA eluent to 1.5 mL or 2.0 mL centrifuge tubes, and tubes are centrifuged (10,000×g) at room temperature for 15 min. The supernatant is discarded[*5]. One microliters of 70% ethanol is used to rinse the DNA pellet. After centrifugation (10,000×g) at 4°C for 5 min, the supernatant is discarded[*5] and pellet is briefly dried. Seventy microliters of 50°C pre-warmed water is added to dissolve the pellet. The concentration and the purity of the extract DNA sample are estimated using the spectrophotometer.

[*1] G2 buffer, QBT buffer, QC buffer and QF buffer are used from the kit, or all buffers can be prepared according to the manufacturer's instructions.

[*2] α-Amylase (high concentration) can be purchased from Nippon Gene Co., Ltd., or the other products showing the equivalent activity can be used.

[*3] RNaseA (100 mg/mL) can be purchased from Qiagen, or the other products showing equivalent activity can be used.

[*4] Proteinase K (20 mg/mL) can be purchased from Qiagen, or other products showing equivalent activity can be used.

1.2. Silica gel membrane type kit method (NIPPON GENE GM quicker 2)

First, 500 mg homogenously ground sample is weighed into a polypropylene tube (15 mL capacity). To the ground sample, 2.1 mL GE1 buffer [*1], 30 µL RNaseA[*1] and 6 µL α-Amylase[*1] are added and mixed in a test tube, then incubated at 37°C for 30 min. During the incubation, the tubes are mixed several times. Then, 60 µL Proteinase K[*1] is added in the sample, mixed to homogenous and incubated at 65°C for 30 min. During incubation, the tubes are mixed several times. Then, 255 µL GE2-K Buffer[*1] is added, mixed well and

incubated for 10 min on ice. Then, the tubes are centrifuged (6,000×g) at 4°C for 15 min. Then, the resulting supernatant is transferred into a 2 mL tube and centrifuged (>13,000×g) at 4°C for 5 min, and the supernatant is discarded. Then, the supernatant is transferred into a 15 mL tube, and 375 μL GB3 buffer[*1] and 375 μL isopropanol are added per 1 mL supernatant. The tube is inverted 10-12 times to mix. Then, 700 μL mixture is placed onto the spin column and tube is centrifuged (>13,000×g) to mix at 4°C for 30 sec, and the supernatant is discarded. This step is repeated until all supernatant is transferred. Then, 650 μL GW buffer[*1] is loaded onto the column, the tube is centrifuged (>13,000×g) at 4°C for 1 min, and the supernatant is discarded. Then, the spin column is transferred to a new 1.5 mL tube, and 55 μL distilled water is loaded directly onto the spin column and incubated at room temperature for 3 min. Then, the tubes are centrifuged (>13,000×g) for 1 min to elute the DNA.

[*1] GE1 buffer, GE2-K buffer, GB3 buffer, GW buffer, RNaseA, α-Amylase and ProteinaseK in the kit (NIPPON GENE GM quicker) are used, or all buffers can be prepared according to the manufacture's instructions.

1.3. Measurement of the DNA purity in the DNA sample solution and preparation and storage of the DNA sample solution

An aliquot of the DNA sample solution is used to dilute using the sterile distilled water, and the absorbances at 260 nm and 280 nm (A260 and A280[*1]) are measured. The DNA concentration is estimated by A260 (1 at 50 ng/μL). If A260/A280 ratio is 1.7 to 2.0, it is considered that DNA is purified substantially[*2]. From the DNA concentration, the DNA sample stock is prepared by diluting to 40 ng/μL using the sterile distilled water. The DNA sample solution is transferred 50 μL per a micro-centrifuge tube and stored frozen at below -20°C. DNA sample solution is used immediately after thawing. The remaining are discarded without saving. When the concentration of the DNA sample stock solution was under 40 ng/μL, the DNA sample solution is directly used without the dilution.

[*1] A260 is used to estimate the DNA concentration, A280 is used to estimate the protein concentration in the sample.

[*2] No further DNA purification is required if the A260/A280 ratio is outside the range of 1.7-2.0.

2. The qualitative real-time PCR (ABI PRISM™ 7900 or 7500)

The insect-resistant genetically modified rice is detected using three real-time PCR methods to detect 63Bt-, NNBt-, CpTI- and rice endogenous gene sequences. For the rice endogenous gene detection, the primers and the probe targeted at the phospholipaseD gene sequences are used. Each primer and probe is dissolved in the sterile distilled water. The sequences of the primers and the probe are as follows.

Primers and probe for detecting the phospholipase D gene:
PLD3959F：5'-GCT TAG GGA ACA GGG AAG TAA AGTT-3'
PLD4038R：5'-CTT AGC ATA GTC TGT GCC ATC CA-3'
PLD-P：FAM-TGA GTA TGA ACC TGC AGG TCGC-TAMRA

Primers and probe for detecting the 63Bt:
T52-SF：5'-GCA GGA GTG ATT ATC GAC AGA TTC-3'
OsNOS-R2：5'- AAG ACC GGC AAC AGG ATT CA-3'

GM63-Taq：FAM-AAT AAG TCG AGG TAC CGA GCT CGA ATT TCCC-TAMRA

Primers and probe for detecting the NNBt:
T52-SF：5'-GCA GGA GTG ATT ATC GAC AGA TTC-3'
OsNOS-R2：5'- AAG ACC GGC AAC AGG ATT CA-3'
NGMr-Taq：FAM-AAT GAG AAT TCG GTA CCC CGA CCT GCA-TAMRA

Primers and probe for detecting the CpTI:
CpTI-2F：5'- TGC AAG TCC AGG GAT GAA GAT-3'
NOS-1R：5'- ACC GGC AAC AGG ATT CAA TC-3'
KDEL-P：FAM- ATG AGA AAG ATG AAC TCT AG-MGB

2.1. Preparation for PCR reaction solution

The total volume of the PCR reaction solution is set 25 μL/well and prepared as follows. After 12.5 μL the Universal PCR Master Mix[*1], 0.4 μL 50 μmol/L primer, 0.25 μL 10 μmol/L probe and 6.45 μL sterile distilled water are mixed, 5 μL 10 ng/μL DNA sample solution is added. As a negative PCR reaction control, an equal volume of the sterile distilled water is added instead of the DNA sample solution[*2]. If 96-well reaction plate is used, the plate is completely sealed[*3] using the sealing applicator. When using ABI7900HT detection system, place the MicroAmp Optical Cover Compression Pad[*4] on the reaction plate.

[*1] Universal PCR Master Mix

Solution prepared using this reagent has a high viscosity; therefore, mixing should be carefully performed. If mixing is not properly performed, the PCR may fail. The Universal PCR Master Mix should be mixed and centrifuged before use. Considering that the reaction solution is not easy to be mixed and centrifuged when Universal PCR Master Mix is contained, it is suggested to pipette all solution at the bottom of the plate.

[*2] Non-Template Control (NTC)

When adding DNA sample solution, the NTC is prepared by adding 2.5 μL water in place of the DNA sample solution.

[*3] 96-well plate, adhesive film and adhesive film applicator

Use the MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.). When sealing the reaction film, refer to the manufacture's instruction.

[*4]MicroAmp Optical Cover Compression Pad (the case of ABI PRISMTM 7900, Thermo Fisher Scientific, Inc.) Do not use in the case of ABI PRISM™ 7500.

2.2. Setting plate information

To start reaction, the plate information (sample position, types and probe characteristics) must be set. The sample ("NTC": Non-Template Control, "UNKN": DNA sample solution) is set according to the positions of the samples prepared. Use "FAM" as a reporter and "TAMRA" as a quencher for the rice endogenous gene, the 63Bt and the NNBt. Use "FAM" as a reporter and "None" as a quencher for the CpTI. In addition, the Passive Reference is set to the "ROX". The run mode is set to the 9600 emulation mode.

2.3. PCR amplification

The plate is set to the instrument, and the reaction is started. The reaction condition is as follows. After pre-heating at 50°C for 2 min and 95°C at 10 min, the thermal cycles are carried out 50 cycles of 95°C for 20 sec and 60°C for 1 min. After making sure that the remaining time is zero when the reaction is terminated, and the analysis on the measurements is performed.

3. Analysis and decision for the result (**see Figure 1**)

For any of the GM rice (63Bt, NNBt, CpTI) or rice positive control detecting, the results are determined based on the confirmation of the exponential amplification curve on the amplification plot and Ct (Cq) value, and clear exponential increasing in the fluorescence intensity from the subject dye (FAM) in the multicomponent. When the exponential amplification curve on the amplification plot is confirmed visually in the GM rice (63Bt, NNBt, CpTI) detection, the samples is suspected to be the GM rice (63Bt, NNBt, CpTI) positive.

Decision for 63Bt:

(1) The Ct (Cq) values of both duplicate sample wells are <48 in both the rice positive control detection and the 63Bt detection (STEP2 pattern1), the sample is 63Bt positive.

(2) If the Ct (Cq) values of both duplicate two sample wells are <48 in the rice positive control detection, but the Ct (Cq) values of all duplicate sample wells are not <48 in the 63Bt detection (STEP2 pattern2), the sample is 63Bt-negative.

(3) If the Ct (Cq) values of both duplicate sample wells are <48 in the rice positive control detection and the Ct (Cq) values of 63Bt detection are neither pattern1 nor pattern2 of STPE2, the DNA is extracted and purified again from the ground sample and tested using the real-time PCR method. If no positive is observed using the second DNA sample preparation, the sample is 63Bt-negative.

The 63Bt-positive pattern

	positive control	63Bt
DNA sample-1	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)

Decision for NNBt:

(1) The Ct (Cq) values of both duplicate sample wells are <48 in both the rice positive control detection and the NNBt detection (STEP2 pattern1), the sample is NNBt positive.

(2) If the Ct (Cq) values of both duplicate two sample wells are <48 in the rice positive control detection, but the Ct (Cq) values of all duplicate sample wells are not <48 in the NNBt detection (STEP2 pattern2), the sample is NNBt -negative.

(3) If the Ct (Cq) values of both duplicate sample wells are <48 in the rice positive control detection and the Ct (Cq) values of NNBt detection are neither pattern1 nor pattern2 of STPE2, the DNA is extracted and purified again from the ground sample and tested using the real-time PCR method. If no positive is observed using the second DNA sample preparation, the sample is NNBt -negative.

The NNBt -positive pattern

	positive control	NNBt
DNA sample-1	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)

Decision for CpTI:

(1) The Ct (Cq) values of both duplicate sample wells are <48 in both the rice positive control detection and the CpTI detection (STEP2 pattern1), the sample is CpTI positive.

(2) If the Ct (Cq) values of both duplicate two sample wells are <48 in the rice positive control detection, but the Ct (Cq) values of all duplicate sample wells are not <48 in the CpTI detection (STEP2 pattern2), the sample is CpTI -negative.

(3) If the Ct (Cq) values of both duplicate sample wells are <48 in the rice positive control detection and the Ct (Cq) values of CpTI detection are neither pattern1 nor pattern2 of STPE2, the DNA is extracted and purified again from the ground sample and tested using the real-time PCR method. If no positive is observed using the second DNA sample preparation, the sample is CpTI -negative.

The CpTI -positive pattern

	positive control	CpTI
DNA sample-1	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)

In addition, if the Ct (Cq) values of all wells were <48 in the rice positive control detection, the DNA is extracted and purified again from the ground sample and tested using the real-time PCR method. If no conclusion is made using the second DNA sample solution, the detection for this sample is considered not testable.

Prior to use different instruments, sample preparation and PCR conditions, the analytical methods should be optimized. Other than the ABI PRISM™ 7900 and 7500, ABI PRISM™ 7700, 7000, etc. can be used. The sensitivity of the real-time PCR method may fluctuate due to the use of different real-time PCR instruments.

Rice (LL601) detection method

In this method, rice and rice products, which have been processed using rice as main ingredient and never been heated, are subjected for testing. The silica gel membrane type kit method (NIPPON GENE GM quicker 2) is used for the DNA extraction and purification. The DNAs are extracted duplicate from a sample in parallel, and the qualitative real-time PCR is carried out using the extracted DNA sample solutions.

1. DNA extraction and purification

1.1. Silica gel membrane type kit method (NIPPON GENE GM quicker 2)

Five hundred milligrams of the homogenously ground sample are weighed into a polypropylene tube (2 mL capacity). To the ground sample, 700 μL GE1 buffer[*1], 10 μL RNaseA[*1], 20 μL proteinase K[*1] and 2 μL α-Amylase are added and mixed for 30 sec to homogeneous[*2]. The sample solution is incubated at 65°C for 15 min. Then, 85 μL GE2-K Buffer[*3] is added, mixed well and incubated for 10 min on ice. And then, the tubes are centrifuged (13,000×g) at 4°C for 5 min. Then, 400 μL supernatant is transferred into a 1.5 mL tube, and 150 μL GB3 buffer and 150 μL isopropanol (100%) are added into the tube. The tube is inverted 10-12 times[*7]. Then, 700 μL mixture is placed to the spin column and the tube is centrifuged (>13,000×g) for 30 sec at 4°C, and the supernatant is discarded. Then, 650 μL GW buffer is loaded onto the column, and the tube is centrifuged (>13,000×g) for 30 sec at 4°C, and the supernatant is discarded. Then, the spin column is transferred to a 1.5 mL tube, and 30 μL distilled water is loaded directly onto the spin column and incubated for 3 min at room temperature. The tubes are centrifuged (>13,000×g) for 1 min to elute the DNAs.

[*1] GE1 buffer, GE2-P buffer, GB3 buffer, GW buffer, RNaseA and ProteinaseK are used from the silica gel membrane type kit (NIPPON GENE GM quicker), or all buffers can be prepared according to the manufacturer's instructions.

1.2. Detection of the DNA purity in the DNA sample solution and preparation for DNA sample stock solution

An aliquot of undiluted DNA sample solution is diluted using the sterile distilled water, and the absorbance of the resulting solution is measured at 260 nm and 280 nm absorbance (A260 and A280[*1]). The DNA concentration is measured at 1 A260 equals to 50 ng/μL DNA. If the A260/A280 ratio is 1.7 to 2.0 showing that the DNA is substantially purified[*2]. From the resulting DNA concentration, the DNA sample stock solution is prepared by diluting to 40 ng/μL using the sterile distilled water. DNA sample solution is transfer 50 μL per a micro-centrifuge tube, and stored frozen at <-20°C. DNA sample solution is used immediately after thawing, the remaining solution is discarded without saving again. When the concentration of the DNA sample stock solution is <40 ng/μL, the DNA sample solution is directly used for the analysis.

[*1] Consider that the A260 absorbance is for the DNA concentration, the A280 absorbance is for the protein in the sample.

[*2] No further DNA purification is required when the A260/A280 ratio is outside the range of 1.7-2.0.

2. The qualitative real-time PCR (ABI PRISM™ 7900 or 7500)

The LL601 is detected using following two real-time PCR methods targeting the LL601 and rice endogenous gene specific sequences. For the rice endogenous gene detection, the primers and the probe that detect the phospholipase D gene sequence are used. Each primer and probe is dissolved in the sterile distilled water. Sequences of primers and probe are as follows.

Primers and probe for detecting phospholipase D gene
F-primer（KVM159）: 5’-TGG TGA GCG TTT TGC AGT CT-3’
R-primer（KVM160）: 5’-CTG ATC CAC TAG CAG GAG GTCC-3’
KVM-P: VIC-TGT TGT GCT GCC AAT GTG GCC TG-TAMRA

Primers and probe for detecting LL601
F-primer（MDB498）: 5’-TAT CCT TCG CAA GAC CCT TCC-3’
R-primer（DPA143）: 5’-ATG TCG GCC GGG CGT CGT TCTG-3’
LL601-P : FAM-TCT ATA TAA GGA AGT TCA TTT CATT-MGB

2.1. Preparation for the PCR reaction solution

The PCR reaction solution is prepared 25 μL/well as follows. After 12.5μL Universal PCR Master Mix[*1], 1 μL 10 μmol/L primer, 0.5 mL 10 μmol/L probe and 5 μL with the sterile distilled water are mixed, 3.0 μL 40 ng/μL DNA sample solution is added. As the negative control PCR reaction, an equal amount of the sterile distilled water is added instead of the DNA sample solution[*2]. If the 96-well reaction plate is used, it is completely sealed[*3] using the sealing applicator. When using the ABI7900HT detection system, the MicroAmp Optical Cover Compression Pad[*4] is placed on the reaction plate.

[*1]Universal PCR Master Mix

This reagent has a high viscosity; therefore, the mixing should be carefully performed. If the mixing is not properly performed, the PCRs may fail. The Universal PCR Master Mix is mixed and centrifuge before use. Considering that the reaction solution is not easily mixed and centrifuged when the Universal PCR Master Mix is contained, it is suggested to pipette all solution at the bottom of the plate.

[*2]Non-Template Control (NTC)

When adding the DNA sample solution, the NTC is prepared by adding 2.5 μL water instead of the DNA sample solution.

[*3]96-well plate, adhesive film and adhesive film applicator

Use the MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.). When sealing the reaction film, refer to the manufacture’s instruction.

[*4]MicroAmp Optical Cover Compression Pad

Use in the case of ABI PRISMTM 7900, do not use in the case of ABI PRISM™ 7500.

2.2. Setting plate information

To start the reaction, the plate information including the sample position, the types and the probe characteristics are set. The sample ("NTC": Non-Template Control, "UNKN": DNA sample solution) is set according to the positions of the samples prepared. Also with respect to the probe characteristics, "VIC" as a reporter and "TAMRA" as a quencher are used to detect the rice endogenous gene. "FAM" as a reporter and "MGB" as a quencher are used to detect the LL601. In addition, the passive reference is set to "ROX". The run mode is set to the 9600 emulation mode.

2.3. PCR amplification

After setting the plate in the instrument, the reaction is started as follows. After pre-heating at 50°C for 2 min

and 95°C at 10 min, the thermal cycles are carried out 45 cycles of 95°C for 15 sec and 60°C for 1 min.

3. Analysis and decision of the result (**see Figure 1**）

For the rice positive control test and the LL601 detection test, the analysis is carried out based on the Ct value obtained from the intersection between the threshold line (Th. line) and the amplification plot. Then, the base line is set between 3-15 cycles at the upper side of the maximum value of the ΔRn noise width, so that the Th. line is set at 0.2 intersecting with the exponential amplification curve. If the Th. line intersects with the amplification curve with noise or the amplification curve without exponential increase, the Th. line is set accordingly so that it does not intersect. The Ct value should be visually confirmed in the amplification plot.

Decision of LL601 contamination:

(1) If the Ct (Cq) values of both duplicate sample wells are obtained at <43 in the rice positive control detection test, and the Ct (Cq) values of all duplicate sample wells are <43 in the GM rice (LL601) detection test (STEP2 pattern1), the sample is the LL601 positive.

(2) If the Ct (Cq) values of both duplicate two sample wells are obtained at <43 in the rice positive control detection, but Ct (Cq) values of all duplicate sample wells are obtained >43 in the GM rice (LL601) detection (STEP2 pattern2), the sample is not contaminated with the LL601 negative.

(3) If the Ct (Cq) values of both duplicate sample wells are obtained at <43 in the rice positive control detection, and the Ct (Cq) values of both GM rice (LL601) detection are neither the pattern1 nor pattern2 of STPE2, the DNAs are extracted and purified again from the ground sample, and the real-time PCR is performed again. If the second DNA sample solution does not improve, the sample is the LL601 negative.

LL601 positive pattern

	positive control	LL601
DNA sample-1	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)

In addition, the Ct (Cq) values of all wells are <43 in the rice positive control detection, the DNAs are extracted and purified again from the ground sample, and the real-time PCR is performed. If the second DNA sample preparation failed, the sample is concluded that it is not testable

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Maize (Bt10) detection method

A qualitative PCR method is applied to the corn kernels or the corn products. For the DNA extraction and purification, the silica gel membrane type kit method (QIAGEN DNeasy Plant Mini Kit) is used. DNA is extracted and purified from one sample in duplicate, and the qualitative PCR method is carried out for each extracted DNA sample solution.

1. DNA extraction and purification

1.1. Silica gel membrane type DNA extraction and purification kit method (QIAGEN DNeasy Plant Mini Kit)

Pre-warmed (65°C) 10 mL AP1 buffer[*1] containing 20 µL RNase A is added to 2 g ground sample in the polypropylene centrifuge tube (50 mL capacity), and vigorously mixed. The solution is heated at 65°C for 15 min while mixing by inverting the centrifuge tube several times. Then, 3,250 µL P3 buffer[*2] is added, and the solution is allowed to stand for 10 min on ice. After incubation, the solution is centrifuged (>4,000×g) at 4°C for 20 min[*3]. Then, 500 µL supernatant is loaded onto the QIAshredder spin column, and centrifuged (>10,000×g) at 4 min, and the eluate is transferred to a centrifuge tube (15 mL capacity). After repeating this step, AW1 buffer[*4] is added 1.5 times the volume of the eluent. The additional 500 µL is loaded onto the mini spin column, and centrifuged at >10,000×g for 1 min[*5]. The remaining mixture is further loaded onto the same mini spin column, centrifuged to discard the eluate under the same conditions. Then, 500 µL AW2 buffer[*6] is loaded, the column is centrifuged (>10,000×g) for 1 min[*5], and the eluate is discarded. This step is repeated three times. The mini spin column is centrifuged (10,000×g) for 20 min or more. The mini spin column is transferred to the centrifuge tube, and 70 µL pre-warmed (65°C) sterile distilled water is added and allowed to stand for 5 minutes, the DNA is eluted by centrifugation (>10,000×g) for 1 min. The distilled water is added again, and the resulting eluates are combined together to have the DNA sample stock solution ready for the analysis.

[*1] AP1 buffer

The silica gel membrane type kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

[*2] P3 buffer

The silica gel membrane type kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

[*3] Supernatant after centrifugation

Check whether the supernatant is clear or not. If it is not clear, the supernatant is centrifuged again under the same conditions.

[*4] AW1 buffer

The silica gel membrane type of kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

[*5] Centrifugation time

Confirm that all liquid is passed through the column appropriately, so that the centrifugation time may vary depending on the sample characteristics.

*6 AW2 buffer
Use the silica gel membrane type of kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

1.2. The DNA purity measurement for the DNA sample and preparation for the DNA sample stock solution

Take an appropriate amount of the DNA sample stock solution, dilute using the sterile distilled water, measure by the ultraviolet absorption spectrum in the range of 200-320 nm, and record the absorbance at 260 nm and 280 nm (A_{260} and A_{280}*2). Calculate the DNA concentration using the absorbance measurements at 260 nm (considering A_{260} 1 = 50 ng/μL).

The A_{260}/A_{280} value of the purified DNA should be in the range of 1.7-2.0. From the resulting DNA concentration, the DNA sample is stored in 20 μL per tube after diluting using the water and frozen below -20°C. DNA sample is used immediately after thawing, and the remaining solution is discarded without re-storing. When the DNA sample stock concentration is under the concentration defined, the sample is directly used for the DNA sample.

2. The qualitative PCR method

The qualitative PCR is done using the targeted DNA and the primer pairs, and analyzed by the electrophoresis*. The Bt10 is detected using the following two tests including the qualitative PCRs targeting the Bt10-specific sequences and the positive control sequences. Nucleotide sequences of the PCR primers are as follows.

· Bt10 detection:
F-primer (JSF5): 5'-CAC ACA GGA GAT TAT TAT AGG GTT ACT CA-3 '
R-primer (JSR5): 5'-ACA CGG AAA TGT TGA ATA CTC ATA CTCT-3 '

· Positive control detection:
F-primer (Zein n-5 '): 5'-CCT ATA GCT TCC CTT CC-3'
R-primer (Zein n-3 '): 5'-TGC TGT AAT AGG GCT GAT GA-3'

*Avoid DNA or DNase contamination into the sample. The method to avoid contamination is referred to the JAS analytical test handbook "Genetically Modified Food Inspection and Analysis Manual for Contamination Prevention Guidelines."

2.1. Preparation for the PCR reaction solution

The reaction solution is prepared to include PCR buffer*1, 0.16 mmol/L dNTP, 1.5 mmol/L magnesium chloride, 0.6 μmol/L 5'and 3' primers, 0.8 units Taq DNA polymerase*2 and 5.0 μL 10 ng/mL DNA sample solution (50 ng DNA). The total volume is added up to 25 μL using the water.
*1 PCR buffer
The PCR buffer II (Thermo Fisher Scientific, Inc., those without magnesium chloride) or one that gives an equivalent result can be used.

*2 Taq DNA polymerase
The AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific, Inc.) or one that gives an equivalent result can be used.

2.2. PCR amplification

The PCR reaction sample is set in the PCR instrument. The reaction conditions are as follows. After incubating at 94°C for 10 min, thermalcyle is repeated 40 times in the following condition per a thermalcycle: 94°C for 25 sec, 62°C for 30 sec, 72°C for 45 sec. After keeping seven minutes at 72°C, the reaction solution is stored at 4°C. As a control reaction, the reaction solution is run without the primer pairs and without the DNA sample solution.
*PCR instrument
Use the GeneAmp PCR System 9700 (Thermo Fisher Scientific, Inc.) or the other instruments that give equivalent results.

2.3 Agarose gel preparation

The PCR solution is analyzed by the agarose-gel electrophoresis, and the PCR products are confirmed by detecting visible band.

2.3.1. Creating an agarose gel

The agarose is weighed and dissolved in the TAE buffer*1 including 5 μL ethidium bromide solution*2 (the final concentration at 10 mg/mL) per 100 mL. The agarose concentration in the agarose gel is set in the range of 1.0-4.0% according to the PCR product size.
*1TAE buffer
Use solution that has a final concentration of 40 mmol/L Tris-acetate and 1 mmol/L EDTA that are adjusted using the water.
*2Pre-staining
The ethidium bromide solution is not necessary added at this stage. The gel can be stained after the electrophoresis according to the section 2.3.3.

2.3.2. Electrophoresis

The electrophoresis is run in the TAE buffer. After mixing the gel loading buffer, 7.5 μL PCR reaction sample is loaded into the wells. The sample is run electrophoresis at 100 V. The electrophoresis is stopped when the bromophenol blue (BPB) in the gel loading buffer reached one-half to two-thirds in the gel.

2.3.3. Gel staining (post-staining)

The following step should be ignored if the agarose gel is pre-stained.

The gel after the electrophoresis is transferred into the container containing the TAE buffer and the agarose gel. Then, the agarose gel is stained using the ethidium bromide solution (10 mg/mL) for 30 min while rocking to mix. Then, the agarose gel is de-stained for 30 min in the TAE buffer without the ethidium bromide solution.

2.4. Gel image analysis

Using the agarose gel imager, the PCR products are visualized under the ultraviolet light (312 nm). Comparing with the DNA size marker, the specific PCR products are analyzed.

2.5. Result

When both 157 bp positive control and 117 bp Bt10 detection bands are detected, the PCR reaction solution is prepared using the identical DNA sample solution, and the PCR is performed using the Bt10 confirmation primer pair[*1,2]. When the 151 bp is detected using the agarose gel electrophoresis, the sample is confirmed to contain Bt10. In the case when the result of the two DNA extracts shows different result, the sample is contaminated with the Bt10. When the positive control primer pair does not show the PCR amplification bands, another agarose gel electrophoresis should perform for confirmation purpose. When the PCR amplification bands at the expected size are still not detected, the result is concluded invalid. In this case, the result from the other extracts should be referred. When no PCR amplification is confirmed using the duplicate DNA extracts, the second DNA extraction is carried out. Subsequently, the result is confirmed by running the PCRs. When the PCR amplification band using a positive control primer pair is not detected, the detection of Maize (Bt10) is not testable. The following table shows the judgement examples.

Judgment examples:

	Sample no.	1	2	3	4	5	6	7	8	9
extract 1	Positive control primers	+	+	+	+	+	+	+	+	-
	Detection primers	+	+	+	+	-	-	+	+	/
	Confirmation primers	+	+	+	+	/	/	-	-	/
extract 2	Positive control primers	+	+	+	-	+	-	+	-	-
	Detection primers	+	+	-	-	-	-	+	-	/
	Confirmation primers	+	-	/	/	/	/	-	/	/
Judgement		Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	/

The second DNA extraction should be carried out in the case of sample no.9 result.

+, positive; -, negative; /, unnecessary test.

[*1] The Bt10 confirmation primer pairs are as follows:

F-primer (Bt10LS-5 '): 5'-GCC ACA ACA CCC TCA ACC TCA -3'

R-primer (Bt10LS-3 '): 5'-GAA GTC GTT GCT CTG AAG AAC AT-3'

[*2] The PCR conditions for the Bt10 confirmation are as follows. After incubating at 94°C for 10 minutes, the thermal cycle (94°C for 25 sec, 30 sec at 65°C and 72°C for 45 sec) is repeated 40 times. After keeping at 72°C for 7 min, the reaction solution is stored at 4°C and used as the PCR product.

Maize (CBH351) detection method

The lateral flow method is used for the maize grain, as well as, the corn grits, the corn flour, the corn meal and the others ("corn semi-finished products".), in which the recombinant proteins are not damaged physicochemically.

For the other maize processed products, the qualitative PCR method is performed.

Note that, the result of the lateral flow method should be confirmed using the qualitative PCR method for the corn semi-finished product.

1. The CBH351detection from the corn kernels

1.1. The lateral flow method

Commercially available kit (Trait Bt9 Corn Grain 5-Minute Test Kit [Part # 7000012], Strategic Diagnostics Inc. [SDI]) can be used. The method is as follows according to the kit instructions. Use of the RO water or the distilled water is highly recommended.

1.1.1. The experimental procedures

After grinding randomly chosen 800 grains, the ground sample* is collected into the 500 mL container with a wide lid. Then, 288 mL water is added and mixed for 10-20 sec. If no supernatant is apparent at this stage, add a small amount of water and mix. Zero point five milliliters of the supernatant is transferred into 1.5 mL-volume sample tube that is provided within the kit, and the Trait Bt9 test strip is set vertically in the sample tube.

*Prepare about 230 g ground samples (If 230 g is not enough from the 800 grains, use the ground 800 grains as the sample).

1.1.2. Analysis of the results

After setting up the test strip in the sample tube, observe the display on the test strip after 5 min. Positive if two red lines are observed on the test strip display, negative if only the control line is observed. If no lines are observed, the test result is invalid.

*Be careful not to read correctly after 5 min since the red lines becomes darker.

2. Detection of the CBH351 from the maize processed products

According to the following qualitative PCR method, the sample is tested duplicate using the DNA sample prepared.

2.1. DNA extraction and purification

2.1.1. Tacos, tortillas, corn chips and corn flakes (limited to those that are heated or processed)

After the freeze-drying, the sample is ground using the food processor. Then, 1 g of the ground sample is collected into the polypropylene centrifuge tube (50 mL size). The DNA is extracted and purified using the ion exchange resin type DNA extraction and purification kit (QIAGEN Genomic-tip 20/G).

In the sample, 4 mL G2 buffer[*1] is added and mixed. Then, 4 mL G2 buffer, 100 μL Proteinase K[*2],

10 μL RNaseA are added and mixed, then the sample is incubated at 50°C for 2 hr. During the incubation, the samples are mixed by inverting several times. Then the sample is centrifuged (>3,000×g) at 4°C for 15 min, the supernatant is transferred into the polypropylene centrifuge tube (15 mL in size) and centrifuged again to spin down the sample. Next, the supernatant is loaded 2 mL at a time onto QIAGEN Genomic-tip 20/G column that is pre-equilibrated using QBT buffer[*1] 1 mL. This step is repeated several times until all supernatant is loaded. Then, the column is washed three times by loading 2 mL QC buffer[*1] at a time onto the column. The column is transferred onto the new centrifuge tube, then the DNAs are eluted by adding 1mL QF buffer[*2] (pre-warmed at 50°C). Transfer the eluate to a centrifuge tube, and mix well with 0.7-fold volume of the isopropyl alcohol, and centrifuge (>10,000×g) at 4°C for 15 min. The supernatant is washed with 1 mL 70% ethanol, and further centrifuged (>10,000×g) at 4°C for 5 min. The supernatant is discarded and the remaining precipitate is dried using the aspirator. One hundred microliters of sterilized distilled water are added, incubated at 65°C for 5 min and performed pipetting to dissolve the DNAs. The resulting solution is used as the DNA sample.

[*1] G2 buffer, QBT buffer, QC buffer and QF buffer are included in the kit. Those solutions can be prepared according to the manufacturer's instructions.

[*2] Use QIAGEN's or those having equivalent efficacy.

2.1.2. DNA extraction and purification from the maize processed products other than those described above

The DNA extraction and purification from the processed food in Notification No. 517 of article 3 by the Ministry of Agriculture, Forestry and Fisheries is done according to "Genetically Modified Food Inspection and Analysis Manual" in JAS Analytical Test Handbook that is prepared by the Food and Agricultural Materials Inspection Center.

2.1.3. Analysis of the DNA purity and preparation for the DNA sample stock solution

Take an appropriate amount of the DNA sample stock solution, dilute using the sterile distilled water, measure the ultraviolet absorption spectrum in the range of 200-320 nm and record the absorbance at 260 nm and 280 nm (A_{260} and A_{280}[*2]). Calculate the DNA concentration using the absorbance measurement at 260 nm (A_{260} 1 = 50 ng/μL).

The A_{260}/A_{280} value of the purified DNA should be in the range of 1.7-2.0. From the resulting DNA concentration, the DNA sample is stored in 20 μL per tube after diluting using water and frozen at <-20°C. DNA sample is used immediately after thawing, the remaining solution is discarded without storing again. When the DNA concentration of the sample stock solution does not meet the concentration required for the PCR, the DNA sample is directly used.

2.2. The qualitative PCR method

The qualitative PCR method includes the PCR amplification of the targeted DNAs using the primer pairs, and analyzed by the electrophoresis[*]. The CBH351 is detected using the two tests including the qualitative PCR targeting the CBH351 and the positive control sequences. Nucleotide sequences of the primers are as follows.

CBH351 detection primer pair:
F-primer (CaM03-5 '): 5'-CCT TCG CAA GAC CCT TCC TCT ATA-3'
R-primer (CBH02-3 '): 5'-GTA GCT GTC GGT GTA GTC CTC GT-3'

Positive control primer pair:
F-primer (Zein n-5 '): 5'-CCT ATA GCT TCC CTT CTT CC-3'
R-primer (Zein n-3 '): 5'-TGC TGT AAT AGG GCT GAT GA-3'

*Avoid DNA and DNase contamination into the sample. The method to avoid contamination is referred to the JAS analytical test handbook "Genetically Modified Food Inspection and Analysis Manual for Contamination Prevention Guidelines."

2.2.1. Preparation of the PCR reaction solution

The reaction solution is prepared in the PCR buffer[*1] including 0.20 mmol/L dNTP, 3 mmol/L magnesium chloride, 0.2 μmol/L 5'and 3' primers, 0.625 units Taq DNA polymerase[*2], 2.5 μL 10 ng/mL DNA sample solution (25 ng DNA). The total volume is adjusted to 25 mL using the water.

[*1] PCR buffer

The PCR buffer II (Thermo Fisher Scientific, Inc., those without magnesium chloride) or use one that gives an equivalent result.

[*2] Taq DNA polymerase

The AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific, Inc.) or use other DNA polymerases that give an equivalent result.

2.2.2. The PCR amplification

The PCR tube is set in the PCR equipment. The reaction conditions are set as follows. After incubating at 95°C for 10 min, the thermalcyle (95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec) is repeated 40 times. After keeping 7 min at 72°C, the reaction solution is stored at 4°C. As a blank reaction, the reaction is run without the primer pairs or without the DNA sample solution. Furthermore, as to confirm the DNA extraction and purification from samples, use a positive control primer pair, instead of the primer pairs for detecting the CBH351.

*The PCR amplification instrument

Use the GeneAmp PCR System 9700 (Thermo Fisher Scientific, Inc.) or the other instruments that give the equivalent results.

2.2.3. The agarose gel electrophoresis

The PCR reaction is analyzed by the agarose-gel electrophoresis, the PCR products are confirmed by detecting the visible band.

2.2.3.1. Creating agarose gels

The agarose is weighed and dissolved in the TAE buffer[*1] including 5 μL ethidium bromide

solution[*2] (10 mg/mL) per 100 mL. The agarose gel concentration is adjusted according to the DNA size of the PCR product of interest.

[*1] TAE buffer

A final concentration is adjusted to 40 mmol/L Tris-acetate and 1 mmol/L EDTA using the distilled water.

[*2]Pre-staining

The ethidium bromide solution is not necessary added at this stage. The gel is stained after the electrophoresis according to the section 2.2.3.3.

2.2.3.2. Electrophoresis

Set the gel in the TAE buffer. After mixing the gel loading buffer, 7.5 μL PCR reaction sample is loaded into the wells. The electrophoresis is performed under 100 V. The electrophoresis is stopped when the bromophenol blue (BPB) in the gel loading buffer ran one-half to two-thirds of the gel.

2.2.3.3. Gel staining (post-staining)

The following step is ignored if the agarose gel was pre-stained.

The agarose gel after the electrophoresis is transferred into the container containing the TAE buffer. Then, the ethidium bromide is added to 10 mg/mL, and the agarose gel is stained for 30 min while rocking on the shaker. Then, the agarose gel is de-stained for 30 min in the TAE buffer without the ethidium bromide.

2.2.4. The gel image analysis

Using the agarose gel imager, the PCR products are visualized as bands under the ultraviolet light (312 nm). Referring to the DNA size markers, the specific PCR product size is analyzed.

2.2.5. Result

When both the 157 bp positive control band and the 170 bp CBH351 detection band are detected, the PCR reaction solution is prepared using the identical DNA sample solution and perform the PCR using the CBH351 confirmation primer pair[*1,2]. When the 171 bp band is detected by the agarose gel electrophoresis, the sample is contaminated with the CBH351. When the duplicate DNA extracts show different result, the sample is judged positive. When the positive control primer pair does not detect the PCR amplicons, another agarose gel electrophoresis is performed. When the PCR amplification bands at the expected size are still not detected, the result is concluded invalid. The result from the other extracts is referred. When no PCR amplifications are confirmed using the duplicate DNA extracts, the second DNA extraction is performed, and the result is confirmed by the PCRs. When the PCR amplification bands using a positive control primer pair is not detected, the detection of the CBH351 is impossible. The following shows the judgment examples.

Judgment examples

	Sample number	1	2	3	4	5	6	7	8	9
extract 1	Positive control primers	+	+	+	+	+	+	+	+	-
	Detection primers	+	+	+	+	-	-	+	+	/
	Confirmation primers	+	+	+	+	/	/	-	-	/
extract 2	Positive control primers	+	+	+	-	+	-	+	-	-
	Detection primers	+	+	-	-	-	-	+	-	/
	Confirmation primers	+	-	/	/	/	/	-	/	/
Judgement		Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	/

The second DNA extraction is performed in the case of sample no. 9 result.

+, positive; -, negative; /, unnecessary test.

* CBH351 confirmed primer pairs are as follows.

F-primer (Cry9C-5 '): 5'-TAC TAC ATC GAC CGC ATC GA-3'

R-primer (35Ster-3 '): 5'-CCT AAT TCC CTT ATC TGG GA-3'

3. Detection of the CBH351 from maize products (corn grits, corn flour, corn meal, etc.)

Without grinding the sample, 230 g sample is tested according to the “1.1. The lateral flow method”. If the sample is judged positive by the lateral flow method, the DNAs are extracted duplicate in parallel according to the "2.1. DNA extraction and purification," then “2.2. The qualitative PCR method” is performed.

The sample is confirmed positive if the 157 bp PCR positive control and the 170 bp PCR amplification bands are detected using the primer pairs for detecting the CBH351.

Maize (DAS59132) detection method

For maize grain, the DNAs are extracted and purified using the silica gel membrane type kit (QIAGEN DNeasy Plant Mini Kit) from a sample in duplicate. The obtained DNA sample is used to test using the following qualitative real-time PCR method. The primers and probe (SSIIb-3 and SSIIb-Taq) for the maize positive control are used to target the maize endogenous gene, *starch synthase IIb* (SSIIb), amplification.

1. DNA extraction and purification

1.1. The silica gel membrane type DNA extraction and purification kit method (QIAGEN DNeasy Plant Mini Kit)

Pre-warmed (65°C) 10 mL AP1 buffer[*1] containing 20 µL RNaseA is added into 2 g ground sample in a polypropylene centrifuge tube (50 mL capacity), and vigorously mixed. The solution is incubated at 65°C for 15 min while rocking the centrifuge tube several times. Then, 3,250 µL P3 buffer[*2] is added, and the solution is allowed to stand for 10 min on ice. After incubation, the solution is centrifuged (>4,000×g) at 4°C for 20 min[*3]. Then, 500 µL supernatant is loaded onto the QIAshredder spin column, and centrifuged (>10,000×g) for 4 min, and the eluate is transferred to a centrifuge tube (15 mL capacity). After repeating this step, the AW1 buffer[*4] is added 1.5 times the volume of the eluent. Additionally, 500 µL is loaded onto the mini spin column, and centrifuged (>10,000×g) for 1 min[*5]. This step is repeated until all is loaded onto the same mini column. The AW2 buffer[*6] 500 µL is then loaded onto the column and the column is centrifuged (>10,000×g) for 1 min[*5], and the eluate is discarded. This step is repeated three times. The column is centrifuged at 10,000×g for >20 min to dry. The mini spin column is transferred to the centrifuge tube, and 70 µL 65°C pre-warmed sterile distilled water is loaded and allowed to stand for 5 min. The DNA is eluted by centrifugation (>10,000×g) for 1 min. The sterile distilled water is added again, and the step is repeated to prepare the DNA sample stock solution.

[*1] AP1 buffer

Use the silica gel membrane type kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

[*2] P3 buffer

Use the silica gel membrane type kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

[*3] supernatant after centrifugation

If the supernatant is not clear, repeat the centrifugation under the same conditions.

[*4] AW1 buffer

Use the silica gel membrane type kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

[*5] Centrifugation time

Confirm that all liquid is passed through the column. The centrifugation time may vary depending on the sample characteristics.

[*6] AW2 buffer

Use the silica gel membrane type kit (QIAGEN DNeasy Plant Mini Kit) or one that is purchased separately.

1.2. Analysis for the DNA purity and preparation for the DNA sample stock solution

Take an appropriate amount of the DNA sample stock solution, dilute using the sterile distilled water, measure the ultraviolet absorption spectrum in the range of 200-320 nm and record the absorbance at 260 nm and 280 nm (A_{260} and A_{280}[*2]). Calculate the DNA concentration using the absorbance measurement at 260 nm (A_{260} 1 = 50 ng/μL).

The A_{260}/A_{280} value of the purified DNA should be in the range of 1.7-2.0. From the resulting DNA concentration, the DNA sample is stored 20 μL per tube after diluting with the water and frozen below -20°C. DNA sample is used immediately after thawing, the remaining solution is discarded without storing it again. When the concentration of the DNA sample stock solution does not meet the concentration defined for the PCR testing, the sample is directly used as the DNA sample.

2. The qualitative real-time PCR (ABI PRISMTM 7900, 7500 or 7700)

2.1. Preparation of the PCR reaction solution

The PCR reaction solution is prepared in 25 μL/well as follows. Twelve point five microliters of the universal PCR Master Mix[*1], 1 μL each primer[*2] (each primer, 10 μmol/L), 0.5 μL probe[*3] (10 μmol/L) are mixed, and the distilled water is added to 20 μL. Then, 5 μL 10 ng/μL DNA sample (50 ng) is added. As a blank, the distilled water is added instead of the DNA sample. After preparation of the reaction solution, the plate is sealed[*4]. The test is done in two wells per a sample, and the PCR reaction solution is prepared for a duplicate test.

[*1] The Universal PCR Master Mix

This reagent has a high viscosity; therefore, mix solely.

[*2] The primers are dissolved in the water.

The sequences for the DAS59132 detection primer pair are as follows.

F-primer (32f): 5'-CCG CAA TGT GTT ATT AAG TTG TCT AAG-3'

R-primer (32r): 5'-GGT GAA TGT CGC CGT GTGT-3'

The sequences for the SSIIb detection primer pair are as follows.

F-primer (SSIIb 3-5 '): 5'-CCA ATC CTT TGA CAT CTG CTCC-3'

R-primer (SSIIb 3-3 '): 5'-GAT CAG CTT TGG GTC CGGA-3'

[*3] Probe is dissolved in the water.

The sequences for the DAS59132 probe are as follows.

5'-FAM-CAA TTT GTT TAC ACC AGA GGC CGA CACG-TAMRA-3 '

The sequences for the SSIIb probe are as follows.

5'-FAM-AGC AAA AGA GCG CTG CAA-TAMRA-3 '

[*4] 96-well plate, film and film applicator

Use the MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.) according to the manufacture's instruction.

[*5] The MicroAmp Optical Cover Compression Pad (Thermo Fisher Scientific, Inc.) is used for the ABI PRISMTM7900.

2. Real-time PCR setting

The amplification of the targeted sequences is detected from the fluorescent reporter "FAM". The quencher of the probe is "TAMRA". The Passive Reference is set using "ROX".

2. 3. PCR amplification

The reaction conditions are as follows. After holding at 50°C for 2 min, the reaction is started at 95°C for 10 min using the hot-start method. One cycle of 95°C for 15 sec and 60°C for 1 min per a cycle is repeated 40 times. Amplification is set using the 9600 emulation mode

3. Analysis of the results (**see Figure 1**)

The results from the DAS59132 and the maize positive control tests are confirmed by the exponential amplification curves and the Ct values from the amplification plot. The FAM fluorescence intensity increase should be confirmed from the multicomponent analysis.

First, when the exponential amplification from the DAS59132 detection test is confirmed visually, doubt that the sample is DAS59132 positive. Then, the threshold value is set at 0.2. The Ct value is obtained at the threshold value.

Use two wells per duplicate DNAs prepared from a sample (total of four wells per a sample test).

The result is confirmed by the following conditions.

(1) The sample is positive when the Ct value <38 from all wells is obtained using the endogenous reference control test, and when the Ct value <38 in all wells using the DAS59132 detection test is obtained.

(2) The sample is negative when the Ct value <38 from all wells is obtained using the endogenous reference control test, and when the Ct value <38 in all wells using the DAS59132 detection test is not obtained.

(3) The sample is negative when the Ct value <38 from all wells is obtained using the endogenous reference control test, and when the Ct value <38 in not all wells using the DAS59132 detection test is obtained. If no positive results are obtained using the second DNA sample prepared, the sample is DAS59132 negative.

The sample is positive when the duplicate tests using the duplicate DNA sample solutions prepared in parallel are all positive (total of four wells per a sample).

Note that the exponential increase in the fluorescence intensity of the FAM should be visually confirmed in analysis of the multicomponent, and no fluorescence intensity change of the ROX or the gradual increase of the FAM fluorescence from the reaction should be confirmed.

Also, when the Ct value <38 in all wells using the maize positive reference control method is confirmed, then repeat "1. DNA extraction and purification" from the sample. If the Ct value <38 is still not obtained, the detection from the sample is not testable

Rapeseed (RT73 *B. rapa*) detection method

This method is designed for testing the rapeseed grain, and the silica gel membrane type kit (NIPPON GENE: GM quicker 2) is used to extract and purify the DNAs. The DNA samples are extracted and purified in duplicate from a sample, and the testing is done using the qualitative real-time PCR method.

The herbicide-tolerant RT73 *Brassica rapa* (RT73 *B. rapa*) is the genetically modified (GM) rapeseed that was developed by crossing the authorized herbicide-resistant RT73 *Brassica napus* (RT73 *B. napus*) and the non-GM rapeseed (*B. rapa*). To detect RT73 *B. rapa*, it is necessary to first identify *B. rapa* and *B. napus*, and then to detect the RT73 in a grain. Therefore, the following method in Section 1, *B. rapa* and the GM rapeseed are detected for the screening purpose, and the single grain detection method in the following Section 2 is used to finalize a decision for the contamination.

1. Screening test

1.1. DNA extraction and purification method

The silica gel membrane type kit method (NIPPON GENE: GM quicker 2) is used to extract and purify the DNAs from the rapeseed. The DNAs are extracted and purified duplicate from each sample, and each DNA sample is tested using the qualitative real-time PCR method. Homogeneously ground 200 mg sample is transferred into 2 mL tube, and 800 µL GE1 buffer[*1], 20 µL Proteinase K and 10 µL RNase A are added and mixed using the vortex mixer[*2]. The resultant sample is incubated at 65°C for 15 minutes. One hundred micro liters of GE2-K buffer[*3,*4] are added and mixed using the vortex mixer. The sample is centrifuged (>13,000×g) at 4°C for 5 min[*5]. Then, 350 µL supernatant[*6] is transferred into a 1.5 mL tube, and 130 µL GB3 Buffer and 130 µL isopropanol are added and mixed by rocking 10-12 times[*7]. After 610 µL mixture is loaded onto the spin column, the mixture is centrifuged (>13,000×g) at 4°C for 30 sec, and the eluate is discarded. Then, 650 µL GW buffer is loaded and centrifuged (>13,000×g) at 4°C for one min, and the eluate is discarded. The spin column is transferred to a new 1.5 mL tube and added 50 µL sterile distilled water and allowed to incubate for three min at room temperature, and the column is centrifuged (>13,000×g) for one minute. The resulting eluate is used for the DNA sample stock solution[*8].

[*1] GE1 buffer

Use buffers in the silica gel membrane type kit (NIPPON GENE: GM quicker 2), or use ones that are purchased separately.

[*2] When the stirring is insufficient, the DNA yield may be significantly reduced. Confirm to vortex vigorously to completely mix. Place the 2 mL tube vertically while firmly stirring for 30 sec. If the mixing is insufficient, vortex more for 30-60 sec.

[* 3] GE2 buffer

Use buffers in the silica gel membrane type kit (NIPPON GENE: GM quicker 2), or use ones that are purchased separately.

[* 4] Even any bubbles are remained in the tube, the GE2-K Buffer can be continuously added. Because the extraction liquid has high viscosity, mix GE2-K buffer sufficiently to make the solution consistent.

[*5] Considering the characteristics of 2 mL tube, set the centrifugal conditions to the maximized g's.

[*6] When recovering the supernatant, be careful not to take any precipitates or floating solids, etc..

[*7] Mix after GB3 buffer and isopropanol are added until the solution becomes clear.

[*8] Prepared DNA sample stock solution to 10 ng/μL using the sterile distilled water, and use as the DNA sample solution.

1.2. The qualitative real-time PCR (ABI PRISMTM 7900 or ABI PRISMTM7500)

Primer pairs and probes used in the qualitative real-time PCR method are as follows. Each primer and probe used is dissolved in the water.

B. rapa identification method

For *B. rapa* identification test, the following a pair of the primers and the probe is used to detect the sequences of the *B. Rapa* acetyl CoA carboxylase (ACCg8) gene and the *B. napus* cruciferin (BnC1) gene.

ACCg8 detection primer pair and probe:
B.rapa-ACCg8 F: 5'-GGT TAT ATA CGG CTT TGT GGT TGC-3 '
B.rapa-ACCg8 R: 5'-AAC ATC AGG CTG TCC AAG AAA GAT-3 '
B.rapa-ACCg8: 5'-VIC-CTA TGT CTG AGG AAT TAT AA-MGB-3 '

BnC1 detection primer pair and probe:
B.napus BnC1-969F: 5'- GAA GCT CTC CTT CGT GGC TAAA-3 '
B.napus BnC1-1043R: 5'- TCA CGA ATT TGA ATC TCG ATA CTCA-3 '
B.napus BnC1-994T: 5'-FAM-ACG TGA ATC TGA TTT TGA-MGB-3 '

RT73 detection method

For rapeseed positive control, the following primers and probe to detect RT73 and acyl-ACP thioesterase (FatA) gene sequences are used.

RT73 detection:
RT73 Primer1: 5'-CCA TAT TGA CCA TCA TAC TCA TTG CT-3 '
RT73 Primer2: 5'-GCT TAT ACG AAG GCA AGA AAA GGA-3 '
RT73 Probe: 5'-FAM-TTC CCG GAC ATG AAG ATC CTC CTT-TAMRA-3 '

FatA detection:
FatA Primer1: 5'-GGT CTC TCA GCA AGT GGG TGAT-3 '
FatA Primer2: 5'-TCG TCC CGA ACT TCA TCT GTAA-3 '
FatA Probe: 5'-VIC-ATG AAC CAA GAC ACA AGG CGG CTT CA-TAMRA-3 '

1.2.1. Preparation for the PCR reaction solution

The PCR reaction solution is prepared in 25 μL per a well. The composition of the solution is as follows: 12.5 μL Universal PCR MasterMix[*1], 0.25 μL target primer pair solution (each primer at 50 μmol/L), 0.5 μL probe solution (10 μmol/L), 22.5 μL sterile distilled water and 2.5 μL DNA sample solution (10 ng/μL)[*2,*3]. Seal plate completely[*4] using the sealing applicator after preparation of the PCR reaction solution, and place the MicroAmp Optical Cover Compression Pad[*5] on the reaction plate. A duplicate test is conducted for each sample.

[*1] The Universal PCR Master Mix

This reagent has a high viscosity. The mixing should be carefully performed. Insufficient mixing may cause the PCR failure. After mixing, centrifuge to keep the solution at the bottom of the sample tube. In addition, considering that stirring and centrifugation are difficult in downstream steps, make sure to dispense all reaction into the wells at the bottom of the well.

[*2] For single grain test, 2.5 μL stock DNA sample solution that is not adjusted for the concentration is used.

[*3] Three types of control samples, 1 well for the Non-Template Control (NTC), 1 well for the *B. rapa* and 2 wells for the *B. napus*. Upon addition of the DNA sample solution, 2.5 μL water in NTC or 2.5 μL standard plasmid for *B. rapa* and *B. napus* is added in each well instead of the sample solution.

[*4] The 96-well plate, the seal and the sealing applicator

Use the MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.). Refer to the manufacture's instruction.

[*5] Use the MicroAmp Optical Cover Compression Pad (in the case of the ABI PRISM 7900, Thermo Fisher Scientific, Inc.). Not applicable to the ABI PRISM 7500.

[*6] For single grain testing, both the *B. rapa* identification and the RT73 detection test are tested in 1 well.

1.2.2. Configuration of the plate information

With respect to the probe characteristics, use "VIC" reporter for the detection ACCg8 and "Non Fluorescent" as quencher, use "FAM" reporter for the detection of BnC1 and "Non Fluorescent" as quencher, use "FAM" reporter for the detection RT73 and "TAMRA" as quencher, use "VIC" reporter for FatA detection and "TAMRA" as quencher. In addition, set the Passive Reference as "ROX" in both the *B. Rapa* identification test and the RT73 detection test.

1.2.3. PCR amplification

The reaction conditions are as follows. After holding at 50°C for 2 min, the reaction is started by the hot-start method, 95°C incubation for 10 min. Then, repeat 40 thermalcycles of 15 sec at 95°C for 1 min and 60°C for 30 sec.

1.2.4. The end-point analysis (ABI PRISMTM 7900)

The end-point analysis is carried out in the *B. rapa* identification test. In [Marker Manager], detectors are set for "ACCg8" and"BnC1". After setting, the Rn value of the ACCg8 and the BnC1 are selected as the fluorescence intensity in [System Table Pane].

1.2.5. The end-point analysis (ABI PRISMTM7500)

The end-point analysis is carried out in the *B. rapa* identification test. In [Select Markers], detectors are set for "ACCg8" and"BnC1". After setting, the Rn value of ACCg8 and BnC1 are selected as fluorescence intensity in [Report] tab.

1.3. Result analysis (**see Figure 2**)

Results are obtained using the DNA samples prepared from a duplicate DNA purification (2 wells tested per a sample in parallel, considering that the duplicate test is conducted per a DNA sample). For *B. rapa* identification test, the result is analyzed by the end-point analysis. For the RT73 detection test, the result is analyzed by the definite amplification curve. For samples that are confirmed to contain *B. rapa* and RT73, the samples must conduct the single grain testing.

From the end-point analysis for the *B. rapa* identification test, if the fluorescence intensity ratio (average of 2 wells) of ACCg8 (VIC) and *B. napus* Positive Control is more than 2.04 using ABI PRISMTM 7900 or more than 1.40 using ABI PRISMTM 7500, the sample is contaminated with the *B. rapa*.

In addition, for the RT73 detection test, the results should be confirmed from the exponential increase in the fluorescent (FAM) intensity and the Ct values in the amplification plot. If the exponential increase in the amplification plot is confirmed, the sample is suspected to have the RT73 contamination. Then, the base line is set at 3-15 cycles at the threshold that is set at 0.2. If the threshold line intersects with the non-specific amplification curve, the threshold line should be set at non-interacting point. The Ct value should be obtained from the threshold line.

For DNA sample solution,

(1) Sample is RT73 positive when the Ct value is obtained under 38 in all wells using both the FatA detection probe (VIC) and the RT73 detection probe (FAM).

(2) Sample is RT73 negative when the Ct value is obtained under 38 in all wells using the FatA detection probe (VIC), but the Ct value is not obtained under 38 in all wells using the RT73 detection probe (FAM).

(3) If the Ct value under 38 using the FatA detection probe (VIC) is not obtained in all wells, the second DNA extraction and purification is conducted. Then, the decision is made according to the protocol following the section "1.2. qualitative real-time PCR." If the result is not consistent using the second DNA sample preparation, sample is "negative."

According to the detection scheme (Figure 3), all results obtained using the duplicate DNA sample prepared in parallel are considered. Samples that are positive for both the *B.rapa* identification test and the RT73 detection test, "2. Single grain testing" is carried out.

For samples positive for the RT73, the exponential increase in the FAM or VIC fluorescence intensity is confirmed by the multicomponent analysis. No decrease in the fluorescence intensity from the ROX and no gradual increase of the FAM and the VIC fluorescent intensity should be detected. The samples showing no signal or Ct value <38 using the FatA probe (VIC) are re-tested. If the Ct value of lower than 38 is still obtained, the detection from this sample is considered impossible.

2. Single grain testing

For samples that are positive for both *B. rapa* and RT73, random seeds are tested by the single grain testing. The DNAs are extracted and purified from each grain and used in "1.2. qualitative real-time PCR method" for the screening test.

2.1. DNA extraction and purification method (single grain extraction)

The silica gel membrane type kit method (NIPPON GENE: GM quicker 96 rapeseed) is used for the DNA extraction and purification from the rapeseed grain. Prior to the testing, the rapeseed is washed as follows. Ten percent SDS solution is added to a beaker containing the rapeseed grains, and stirred using a spatula. Repeat this step three times. Then, the grains are rinse using water in a beaker. Repeat this step three times. After washing and rinsing, the single rapeseed grains are placed in each well in the grinding plate (RCD-96) and dried at 65°C for 1 hr. Once the rapeseed is dried, place a metal cone (MC-96415R) in the CPD-96, grind at 1,500 rpm for 20 sec using the MULTI-BEADS SHOCKER (YASUI KIKAI)[*1]. After grinding, add 500 µL GE1 buffer[*2], 20 µL Proteinase K, 10 µL RNaseA and mix for 15 sec at 1,500 rpm using the MULTI-BEADS SHOCKER (Yaui kikai, Japan). Incubate the plate at 65°C for 15 min[*3]. Add 85 µL GE2-K buffer[*4,5] and mix for 15 sec at 1,500 rpm using the MULTI-BEADS SHOCKER. Centrifuge for 5 min at 2,900 rpm using the METALFUGE (YASUI KIKAI MBG 100). Then, 400 µL supernatant[*6] is added to the filter plate[*7], and centrifuged for 5 min at 2,900 rpm using the METALFUGE. After adding 150 µL GB3 Buffer and 150 µL isopropanol per well, the samples are mixed by pipetting[*8]. Seven-hundred microliter sample is loaded onto the spin-column plate that is pre-set on the collection plate, and centrifuge at 2,900 rpm for 5 min using the METALFUGE, discard the eluate collected in the collection plate. Then, 650 µL GW buffer is loaded and centrifuged for 5 min at 2,900 rpm using the METALFUGE, then discard the eluate. To remove the ethanol remained completely, the spin-column plate is centrifuged at 2,900 rpm for 20 min using the METALFUGE. The spin column plates are transferred to a new collection plate, and incubate for 3 min at room temperature. Fifty microliter water is added, incubated for 3 min, centrifuged for 5 min at 2,900 rpm using the METALFUGE. The resulting eluate is used as the DNA sample solution .

[*1] Spin-down for one minute at 2,900 rpm using the METALFUGE (YASUI KIKAI) after grinding to drop the sample attached to the lid and to prevent from contamination.

[*2] GE1 buffer

Use one that comes with the silica gel membrane type kit (NIPPON GENE: GM quicker 96), or use one that has been purchased separately.

[*3] During the incubation at 65°C, the air in the well could expand and there is a possibility for the contamination. In order to prevent this, the lid should be tightly sealed and covered with the Saran Wrap.

[*4] GE2 buffer

Use one with the silica gel membrane type kit (NIPPON GENE: GM quicker 96), or use one that is purchased separately.

[*5] To prevent contamination into the other wells, spin-down for one minute at 2900 rpm using the

METALFUGE (YASUI KIKAI) before opening the lid. Mix sorely so that the sample with the GE2-K buffer becomes uniform.

*6 Supernatant is recovered while no precipitates and solids are lost.

*7 The polypropylene filter capacity 800 μL, pore size of 0.45 μL Whatman is used for the filter plate.

*8 Add the GB3 buffer after adding isopropanol, and then mix sorely. If any debris are confirmed, the sample is mixed until the solution becomes clear.

2.2. Analysis of the results (**see Figure 2**)

Test result on each DNA sample stock solution is analyzed based on the end-point analysis for *B. rapa* identification and a clear amplification curve for the RT73 detection test. The RT73 *B. rapa* is present if the sample is positive for both the *B. rapa* and RT73 tests.

For end-point analysis of the *B.rapa* identification test, the DNA sample stock solution is *B. rapa* positive if the fluorescence intensity ratio (average value of two wells) of the ACCg8 (VIC) of the DNA sample stock solution and the fluorescence intensity of the ACCg8 (VIC) of *B.napus* positive control is more than 2.63 (in the case of using ABI PRISMTM 7900) or more than 1.69 (in the case of using ABI PRISMTM 7500). The RT73 is present in the DNA sample stock solution if the fluorescence intensity (the average value of two wells) of BnC1 (FAM) and *B.napus* positive control is less than 0.28 (in the case of using ABI PRISMTM 7900) or 0.35 (in the case of using ABI PRISMTM 7500). For the RT73 detection test, the exponential increase of the fluorescence (FAM) intensity in the Amplification plot should also be confirmed.

First, the RT73 positive is confirmed when the exponential amplification curve in the Amplification plot is observed. Then, the base line should be set (from 3-15 cycles) at the upper side of the maximum value of the ΔRn noise. The threshold line (Th. Line) is set at 0.2 at the exponential amplification of the curve. However, if the Th. line intersects with the noise or amplification curve, then the Th. Line is placed accordingly so that no curves are intersected. Using the FatA detection method (VIC) and the RT73 detection method (FAM), the RT73 is considered contaminated if the Ct value is <38. The RT73 is not contaminated if the Ct value <38 is obtained from FatA detection probe (VICs) and is not obtained from the RT73 detection probe (FAM). Also, if the Ct value is obtained more than 1.3 times the case of the FatA using the RT73 detection probe (FAM), the sample should be considered that the RT73 is not contaminated. Note that the samples with the RT73 positive are confirmed by the multicomponent analysis, the exponential increase in the fluorescence intensity of FAM or VIC and no clear change in the fluorescence intensity by the ROX. Also, the DNA sample stock solution having the Ct value <38 in the FatA detection test should be confirmed by the qualitative real-time PCR method. If the result is not confirmed, the testing is considered impossible. If Ct value of less than 38 using the FatA detection probe (VIC) is confirmed in more than 90 grains out of 92 grain, the sample is testable. If the Ct value less than 38 is obtained from less than 89 grains in the FatA detection, the sample is considered not testable. In this case, "2.1. DNA extraction and purification method (single grain extraction)" or later should be performed again from randomly selected 92 grains.

If there is one grain positive for the *B. rapa* and the RT73 detection method, the sample is considered to contain the RT73 *B. rapa*.

Papaya (PRSV-YK, PRSV-SC, PRSV-HN) detection method

In this method, the fresh and the processed papayas are tested, and the ion exchange resin type kit method (QIAGEN Inc. Genomic-tip 100 / G) is used for the DNA extraction and purification.

As an alternative to the low-degree processed papayas, such as fresh and dried papayas*, the silica gel membrane type method (QIAGEN DNeasy Plant mini) is used for the DNA extraction and purification.

The DNAs are extracted in duplicate from a sample in parallel, and the qualitative real-time PCR is carried out using the extracted DNA sample solutions.

* Repeat until a sufficient DNA amount is extracted and purified.

1. The DNA extraction and purification from the fresh and the processed papayas

Fresh papaya and processed papayas are categorized into the following food forms, then the sample pretreatment is carried out prior to the DNA extraction and purification accordingly to the protocol shown below.

① Fresh and seasoned pickled products (sample that is not dried and partially holds original papaya shape, such as the fresh, pickled and canned papaya)
② Dried foods products (dried papaya)
③ Candied dried products (dried fruit)
④ Dried products (health food, tea, etc)
⑤ Pulp-containing gel-like products (jam, puree, etc)
⑥ Fruit juice and beverage products (fruit mixed juice, tonic drink, etc.)
⑦ Iced products (ice, sherbet, etc)

1.1. Sample pretreatment

1.1.1. Fresh and seasoned pickled products

From the products, only those that are papayas (for fresh papaya, only the sarcocarp part excluding the seed and the pericarp) are sampled visually and used. The samples are rinsed three times with the sterile distilled water using twice the volume of the products. After wiping off the excess water, the samples are ground using the food processor (For the fresh papaya, grind without rinsing). Ten-grams ground sample is weighed in a polypropylene centrifuge tube (50 mL capacity), and 30 mL G2 buffer* is added and mixed to homogeneous.

1.1.2. Dried foods products

From the products, only those that are papayas (for fresh papaya, only the sarcocarp part excluding the seed and the pericarp) are sampled visually and used. The samples are ground using the food processor. Two-grams of the ground sample are sampled into a polypropylene centrifuge tube (50 mL capacity), and 30 mL G2 buffer* is added and mixed to homogeneous.

1.1.3. Candied dried products

From the products, only those that are papayas (for fresh papaya, only the sarcocarp part excluding the seed and the pericarp) are sampled visually and used. The samples are rinsed three times with the sterile distilled water using twice the volume of the products. The samples are ground using the food processor (For the fresh

papaya, grind without rinsing). Ten grams of the ground sample are weighed in a polypropylene centrifuge tube (50 mL capacity), 30 mL G2 buffer* is added and mixed to homogeneous.

1.1.4. Dried products

Two grams of ground and homogenized samples using the food processor are sampled into a polypropylene centrifuge tube (50 mL capacity), 30 mL G2 buffer* is added and resulting solution is mixed well to homogeneous.

1.1.5. Pulp-containing gel-like product

Ten grams of the ground samples are weighed in a polypropylene centrifuge tube (50 mL capacity), 30 mL G2 buffer* is added and the resulting solution is mixed well to homogeneous.

1.1.6. Fruit juice and beverage products

The product is homogenized and mixed well prior to opening. One hundred milliliters from the sample is sampled using the graduated cylinder, transferred to a freeze-dried container (500 mL capacity) and placed in the -80°C freezer while making the container tilted. Then, the container is set in the freeze-drying machine, and 30 g dried sample is transferred to the mortar and dissolve in 20 mL G2 buffer* using the pestle. Then the whole sample solution is transferred into a polypropylene centrifuge tube (50 mL capacity), and the remaining sample is collected using 10 mL G2 buffer*, then the sample solution is mixed well to homogeneous by inverting the tube.

1.1.7. Iced products

One hundred grams of the sample are weighed in the freeze-dried container and freeze-dried for 24 hours. Then, 10 g sample is added gradually into a polypropylene centrifuge tube containing 30 mL G2 buffer*, and dissolve to homogeneous by inverting the tube.

*Use the G2 buffer that comes with the Qiagen kit (Cat. No. 19060), or solution that is made according to the manufacturer's instructions.

1.2. DNA extraction and purification from the papaya sample

1.2.1 DNA extraction and purification

1.2.1.1 Ion exchange resin type DNA extraction and purification kit (QIAGEN Genomic-tip 100/G) method

In the homogenized samples, 500 µL cellulose and 20 µL 100 mg/ml RNaseA*4 (add 20 µL α-Amylase*2 for jam product only) are added and mixed to homogenous, and incubated for 1 hr at 50°C. During the incubation, the tubes are mixed several times. Then, 200 µL Proteinase K*3 is added into the sample and mixed to homogenous, and the sample is incubated at 50°C for 1 hr. During the incubation, the tubes are mixed several times. Then, the samples are centrifuged (3,000×g) at 4°C for 20 min. The resulting supernatant (about 25-35 mL) is transferred into the QIAGEN Genomic-tip 100/G, which is pre-equilibrated using 4 mL QBT buffer*1. Then, the column is washed using 7.5 mL QC buffer*1 three times, and the column is transferred to a new centrifuge tube, loaded 2 mL QF buffer*1, which was pre-warmed to 50°C, to elute the DNAs. An equal volume of isopropanol is added to eluate the DNAs and mixed solely. Then, transfer to new 1.5 mL or 2.0 mL centrifuge tubes, and the tubes are centrifuged (10,000×g) for 15 min at room temperature. The resulting supernatant is discarded*5. One milliliters of 70% ethanol are used to rinse the pellet. After centrifugation

(10,000×g) at 4°C for 5 min, the supernatant is discarded[*5], and the pellet is dried. Seventy microliters of 50°C pre-warmed water is added to dissolve the pellet. The concentration and the purity of the extract DNA sample are estimated using the spectrophotometer.

[*1] G2 buffer, QBT buffer, QC buffer and QF buffer are used from the kit, or all buffers can be prepared according to the manufacturer's instructions.

[*2] α-Amylase (high concentration) can be purchased from Nippon Gene Co., Ltd., or the other products having the equivalent activity can be used.

[*3] Proteinase K (20 mg/mL) can be purchased from Qiagen, or the other products having equivalent activity can be used.

[*4] RNaseA (100 mg/mL) can be purchased from Qiagen, or the other products having equivalent activity can be used.

[*5] When precipitate is not dissolved, it is dissolved at 65°C while mixing for 15 min. If the precipitate is not completely dissolved, centrifuge (12,000×g) at 4°C for 3 min. The resulting supernatant is transferred to a new centrifuge tube.

1.2.1.2 Silica gel membrane type kit method (QIAGEN DNeasy Plant Mini)

The sarcocarp excluding seeds from the papaya is cut into about 10 mm block, and lyophilized. Next, the samples are mixed, and ground using the food processor. The DNAs are extracted and purified from the ground sample according to following method.

The sample material 80 mg is weighed into 2 mL microcetrifuge tubes. Sixty microliter 50°C pre-warmed AP1 Buffer and 4 μL RNaseA[*1] are added to the sample and mixed to homogenous, incubated for 15 min at 65°C. During incubation, the tubes are mixed several times. Then, 195 μL P3 Buffer is added, incubated for 5 min on ice, and the sample is centrifuged (10,000×g) at room temperature for 5 min. The resulting supernatant is transferred to the QIAshredder spin column, centrifuged (10,000×g) at room temperature[*2] for 5 min, and the flow-through is transferred into a new centrifuge tube (1.5 mL or 2.0 mL capacity) without disturbing the pellet. Then, 1.5 volume of AW1 Buffer is added to the cleared lysate, and mixed for 10 sec by the vortex mixer. Subsequently, the lysate is transferred into the mini spin column 500 μL volume at a time and centrifuged (10,000×g) at room temperature for 5 min. The resulting supernatant is discarded. This step is repeated until all of the lysate is centrifuged. Then, 500 μL AW2 buffer is added to the column, centrifuged (10,000×g) at room temperature for 5 min. After discarding the flow-through fraction, this step is repeated and centrifuged (10,000×g) at room temperature for 15 min to dry the column. The column is transferred to the centrifuge tube, then 50 μL 50°C pre-warmed distilled water is loaded directly onto the column membrane, incubated at room temperature for 5 min. Then, the column is centrifuged (10,000×g) for 1 min to elute the DNAs. The same operation is repeated to increase the yield.

[*1] RNaseA (100 mg/mL) can be purchased from Qiagen (Cat. No. 1018048), or the other products having equivalent activity can be used.

[*2] When the precipitates are mixed in the mixture, the column tends to get clogged. In this case, the centrifugation time is extended up to about 10 min in order to complete elution.

1.2.2. Detection of the DNA purity and preparation for the DNA sample stock solution

An aliquot of the undiluted DNA sample solution is diluted using the sterile distilled water, and the absorbance of the resulting solution is measured at 260 nm and 280 nm absorbance (A260 and A280[*1]). The DNA concentration is measured using A260 (1 A260 equal to 50 ng/μL DNA). If the A260/A280 ratio is 1.7 to 2.0, the DNA is purified substantially[*2]. From the resulting DNA concentration, the DNA sample solution is prepared by diluting to 50 ng/μL using the sterile distilled water. DNA sample solution is transferred 40 μL per a micro-centrifuge tube, and stored frozen at <-20°C. The DNA sample stock is used immediately after thawing, the remaining solution is discarded without saving again. When the concentration of the DNA sample stock solution is under 10 ng/μL, the DNA sample solution is directly used.

[*1]Considered A260 as the absorbance measured for the DNA concentration, A280 as the absorbance measurements for protein in the sample.
[*2]No further DNA is purified when the A260/A280 ratio is outside the range of 1.7-2.0.

2. Qualitative real-time PCR (ABI PRISM™ 7900 or 7500)

For the PRSV-YK and the PRSV-SC detection, the primers and the probe for detecting the boundary region of the *Papaya Ringspot Virus* coat protein (PRSV-cp) gene that is specifically introduced into each GM lines and *Cauliflower mosaic virus* 35S promotor are used. For the PRSV-HN detection, the primers and the probe for detecting the boundary region of the papaya genome and GM line specific sequences are used. For *cauliflower mosaic virus* 35S promotor detection, the primers and probe set (CaM) is used. For the positive control papaya detection, the primers and the probe for detecting the papaya endogenous gene are used. Each primer or probe is dissolved in the sterile distilled water. The sequences of the primers and the probe are as follows.

Primers and probe for detecting the PRSV-YK:
YK-2F: 5'-ACA CGG GGG ACT CTA GAG -3'
YK-2R: 5'-ACC GGT ATC CAC AGC TTC -3'
YK-2P: 5'-FAM- TCC CTT CCA TGG CGTC-TAMRA-3'

Primers and probe for detecting the PRSV-SC:
SC-F: 5'-CAT TTC ATT TGG AGA GAA CACG-3'
SC-R: 5'-ACC AGC ATC CAC AGC TTC-3'
SC-P: 5'-FAM-ACT CTA GAG GAT CCA TGT CCAA-TAMRA -3'

Primers and probe for detecting the PRSV-HN:
HN-F: 5'-GAC GAG TAC AAG GAG ACG CC-3'
HN-R: 5'-GTT GTC ACT GAA GCG GGA AG-3'
HN-P: 5'-FAM-TGG CTG CTA TTG GGC GAA TCA ACT AC-BHQ1-3'

Primers and probe for detecting the CaM:
35S-F：5'-GCC TCT GCC GAC AGT GGT -3'
35S-R：5'-AAG ACG TGG TTG GAA CGT CTTC-3'
35S-P：5'-FAM- CAA AGA TGG ACC CCC ACC CACG-TAMRA-3'

Primers and probe for detecting the papaya endogenous gene:
Q-Chy-1F2: 5'-CCA TGC GAT CCT CCCA-3'
Q-Chy-2R: 5'-CAT CGT AGC CAT TGT AAC ACT AGC TAA-3'
Q-Chy-P(new): 5'-FAM-TTC CCT TCA TCC ATT CCC ACT CTT GAGA-TAMRA-3'

2.1. Preparation of PCR reaction solution

The PCR reaction solution is set 25 μL/well and prepared as follows. After 12.5 μL TaqMan Gene Expression Master Mix [*1], 0.4 μL 50 μmol/L primer, 0.25 μL 10 μmol/L probe and 6.45 μL sterile distilled water are mixed,

5 μL DNA sample solution is added. As a negative control PCR reaction, an equal amount of the sterile distilled water is added instead of the DNA sample solution[*2]. If the 96-well reaction plate is used, it is completely sealed using the sealing applicator[*3]. If the ABI7900HT detection system is used, place the MicroAmp Optical Cover Compression Pad[*4] on the reaction plate. Each DNA sample solution is tested duplicate using the PRSV-YK, the PRSV-SC, the PRSV-HN and the papaya positive control detection methods.

[*1] TaqMan Gene Expression Master Mix or EagleTaq Master Mix with ROX

The solution prepared using this reagent has high viscosity; therefore, mixing should be carefully performed. If mixing is not properly performed, the PCR may fail. The Master Mix should be mixed and centrifuge before use. Considering that the reaction solution is not easy to be mixed and centrifuged when the Master Mix is contained, it is suggested to pipette all solution at the bottom of the plate.

[*2] Non-Template Control (NTC)

Upon addition of the DNA sample solution, the NTC is prepared by adding 2.5 μL water instead of the DNA sample solution.

[*3] 96-well plate, adhesive film and adhesive film applicator

Use the MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.). When sealing the reaction film, refer to the manufacture's instruction.

[*4] Use the MicroAmp Optical Cover Compression Pad in the case of the ABI PRISMTM 7900. Do not use in the case of ABI PRISM™ 7500.

2.2. Setting plate information

To start reaction, the plate information (sample position, types and probe characteristics) must be set. Also with respect to the probe characteristics, "FAM" as a reporter and "TAMRA" as a quencher are used for the probes (YK-2P, SC-P, HN-P, 35S-P and Q-Chy-P [new]). In addition, the passive reference is set to the "ROX". The run mode is set to the 9600 emulation mode.

2.3. PCR amplification

The plate is set to the instrument, and the reaction is started. The reaction condition is as follows. After pre-heating at 50°C for 2 min and 95°C at 10 min, the thermal cycles are carried out 45 cycles of 95°C for 15 sec and 60°C for 1 min. After making sure that the remaining time is zero when the reaction is terminated, the results are analyzed.

3. Analysis and decision of the results (**see Figure 3**)

For any of the three types of the GM papaya line (PRSV-YK, PRSV-SC or PRSV-HN) detection, the CaM or the papaya positive control, the results are analyzed based on the confirmation of the exponential amplification curve in the amplification plot and the Ct (Cq) values at threshold (Th) value of 0.2 and the definite exponential increase in the fluorescence intensity from the dye (FAM). When the exponential amplification curve on the amplification plot is confirmed visually from both of the GM papaya and the CaM detections, the samples are suspected the GM papaya-positive.

Decision for PRSV-YK:

(1) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection

and the Ct (Cq) values of all duplicate sample wells are less than 43 (STEP2 pattern1) in both the GM papaya detection and the CaM detection, the sample is found PRSV-YK-positive.

(2) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection, but the Ct (Cq) values of all duplicate sample wells are not less than 43 (STEP2 pattern1) in both the GM papaya detection and the CaM detection (STEP2 pattern2), the sample is found PRSV-YK-negative.

(3) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection, and Ct (Cq) values of both GM papaya detection and CaM detection are neither pattern1 nor pattern2 in STEP2, the DNAs are extracted and purified again from the ground sample, and the real-time PCR detection is performed accordingly. Consequently, a decision for the sample is made. If the positive result is not obtained using the second DNA sample solution, the sample is found PRSV-YK-negative.

The pattern of PRSV-YK result

	positive control (Chy)	PRSV-YK	CaM
DNA sample-1	(+/+)	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)	(+/+)

Decision for PRSV-SC:

(1) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection and the Ct (Cq) values of all duplicate sample wells are less than 43 (STEP2 pattern1) in both the GM papaya detection and the CaM detection, the sample is found PRSV- SC -positive.

(2) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection, but the Ct (Cq) values of all duplicate sample wells are not less than 43 (STEP2 pattern1) in both the GM papaya detection and the CaM detection (STEP2 pattern2), the sample is found PRSV- SC -negative.

(3) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection, and Ct (Cq) values of both GM papaya detection and CaM detection are neither pattern1 nor pattern2 in STEP2, the DNAs are extracted and purified again from the ground sample, and the real-time PCR detection is performed accordingly. Consequently, a decision for the sample is made. If the positive result is not obtained using the second DNA sample solution, the sample is found PRSV- SC -negative.

The pattern of PRSV-SC result

	positive control (Chy)	PRSV-SC	CaM
DNA sample-1	(+/+)	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)	(+/+)

Decision for PRSV-HN:

(1) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection and the Ct (Cq) values of all duplicate sample wells are less than 43 (STEP2 pattern1) in both the GM papaya detection and the CaM detection, the sample is found PRSV- HN -positive.

(2) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection, but the Ct (Cq) values of all duplicate sample wells are not less than 43 (STEP2 pattern1) in both the GM

papaya detection and the CaM detection (STEP2 pattern2), the sample is found PRSV- HN-negative.

(3) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection, and Ct (Cq) values of both GM papaya detection and CaM detection are neither pattern1 nor pattern2 in STEP2, the DNAs are extracted and purified again from the ground sample, and the real-time PCR detection is performed accordingly. Consequently, a decision for the sample is made. If the positive result is not obtained using the second DNA sample solution, the sample is found PRSV- HN -negative.

The pattern of PRSV-HN result

	positive control (Chy)	PRSV-HN	CaM
DNA sample-1	(+/+)	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)	(+/+)

In addition, if the result of a sample in all wells using the papaya positive control detection are <43 Ct (Cq) values, the DNAs are extracted and purified again from the ground sample. Then, the real-time PCR is performed. If the second DNA sample solution does not give a conclusion, the testing of the sample is concluded not testable.

Potato (F10, J3) detection method

In this method, the fresh and the processed potatoes are tested, and the potato DNAs are extracted in duplicate from a sample in parallel, and the qualitative real-time PCRs for F10 and J3 detection are carried out using the prepared DNA sample solutions.

4. DNA extraction and purification

The foods containing potato are processed in various forms. This method describes an example for the sample pretreatment and the DNA extraction and purification steps from the fresh potatoes (potato tuber part that is used for food in general) using the ion exchange resin type DNA extraction and purification kit* (QIAGEN Genomic-tip 100/G).

* Not only QIAGEN Genomic-tip 100/G but also the other kits having equivalent performance can be used.

4.1. Sample pretreatment

The potato tuber is rinsed well using water and peeled, and then about 30 g parts around the dormant bud is isolated. For the processed potato, only those that are potatoes are isolated. The samples are ground to homogenous using the food processor*1, and the DNA is extracted and purified*2.

*1 To prevent contamination, all instruments for the sample pretreatment are used free of the DNA contaminants. The DNA-ZAP (Amibon) or other reagents having the equivalent activity can be used.

*2 Any remaining ground samples are transferred into the polypropylene centrifuge tube (50 mL capacity) and stored frozen at -20°C.

4.2. DNA extraction and purification

1.2.1 Ion exchange resin type DNA extraction and purification kit method (QIAGEN Genomic-tip 100/G)

Into the 8 g homogenized samples*1 in 50 mL tube, 20 mL G2 buffer*2, 20 μL α-amylase*3, 20 μL RNaseA*4 and 500 μL cellulose*5 are added and mixed vigorously to homogenous. The sample is incubated at 50°C for 1 hr. During incubation, the tubes were mixed several times. Then, 200 μL Proteinase K*3 is added and incubated at 50°C for 1 hr. During incubation, tubes are mixed several times. Then, the sample is centrifuged (3,000×g) at 4°C for 20 min. The obtained supernatant is loaded onto the QIAGEN Genomic-tip 100/G, which is pre-equilibrated using 4 mL QBT buffer*1. Then, the column is washed using 7.5 mL QC buffer*2 three times, and the column is transferred to a new centrifuge tube. Then 2 mL QF buffer*2 is loaded, and the DNA is eluted. Three milliliters of isopropanol are added and mixed well. Then, the solution is transferred to four to six centrifuge tubes, and the tubes are centrifuged (13,000×g) at 4ºC for 20 min. After discarding the supernatant, 1 mL 70% ethanol is added and centrifuged (13,000×g) at 4°C for 10 min. The supernatant is discarded and the pellet is dried. Fifty-five microliters water is added to dissolve the pellet, and sequentially dissolve the pellet to have the extracted and purified DNA sample ready for the detection.

*1 Potato flour and its processed products have the low DNA content. If the prepared DNA sample solution is <10 ng/μL, the DNA sample is prepared again. Total DNA sample solution (approximately 110 μL), repeat ethanol precipitation to concentrate in 55 μL water. If the DNA concentration is still <10 ng/μL, the DNA sample solution is used directly for the test.

*2 G2 buffer, QBT buffer, QC buffer and QF buffer that come with the kit are used, and all of these buffers can be prepared according to the manufacture's manual.

*2 α-Amylase (high concentration) can be purchased from Nippon Gene Co., Ltd., or other products having the equivalent activity can be used.

*3 Proteinase K (20 mg/mL) can be purchased from Qiagen, or other products having equivalent activity can be used.

*4 RNaseA (100 mg/mL) can be purchased from Qiagen, or other products with equivalent activity can be used.

[*5] When the precipitate does not dissolve, the sample solution can be incubated at 65°C while rocking for 15 min. If the precipitate does not be completely dissolve, the sample solution is centrifuged (12,000×g) at 4°C for 3 min, and the resulting supernatant is transferred to a new centrifuge tube.

4.2.2. Measuring the DNA purity in the DNA sample solution and preparation of the DNA sample stock

An aliquot of the DNA sample solution is diluted using the sterile distilled water, and the absorbance of the resulting DNA solution is measured at 260 nm and 280 nm (A260 and A280) [*1]. The DNA concentration is measured using A260 as 50 ng/μL DNA. If the A260/A280 ratio is 1.7 to 2.0, the DNAs have been purified substantially[*2]. From the resulting DNA concentration, the DNA sample stock solution is prepared by diluting the original DNA sample solution to 50 ng/μL using the sterile distilled water. The DNA sample solution is stored 40 μL per a tube and frozen at <-20°C. DNA sample solution is used immediately after thawing, the remaining solution is discarded without saving again. If the concentration of the original DNA sample solution is under 10 ng/μL, it is directly used for the test.

[*1] A260, the absorbance measurement for the DNA concentration; A280, the absorbance measurement for the protein contaminants in the sample.

[*2] No further DNA purification is required when the A260/A280 ratio is outside the range of 1.7-2.0.

5. Qualitative real-time PCR (ABI PRISMTM 7900 or 7500)

For the GM potato (F10, J3) detection, the primers and the probe that detect the boundary region between the GM potato (F10, J3) genome and the sequences introduced into the event are used. For potato positive control detection, the primers and the probe to detect the potato endogenous gene, *Adenine Phosphoribosyl Transferase* (APRT), are used. Each primer or probe is dissolved in sterile distilled water. Sequences of primers and probe are as follows.

Primers and probe for detecting F10:
F10 F: 5'-GAAGCTATAACAATAACTGGTCC-3'
F10 R: 5'-CACACACTTCGTTTACAC-3'
F10 P: 5'-FAM-TATATATCCTGCTGGACCAGTTG-TAMRA-3'

Primers and probe for detecting J3:
J3 F: 5'-ATCAAAACCGGTACTCAAATTT-3'
J3 R: 5'-GAGTTGTCAAATGTGAATTTATTTC-3'
J3 P: 5'-FAM-CAACAGGACAACCACAAGCTAGGAAACTCAC-TAMRA-3'

Primers and probe for detecting APRT:
APRT F: 5'-TGAAAACGATCCCGATCG-3'
APRT R: 5'-CAATCCCAGCGATACGTTC-3'
APRT P: 5'-FAM-TGGCGCCTCATGATCCG-TAMRA-3'

5.1. Preparation of the PCR reaction solution

Total volume of the PCR reaction solution is set 25 μL/well and prepared as follows. After 12.5 μL TaqMan Gene Expression Master Mix[*1], 0.4 μL 50 μmol/L primer, 0.25 μL 10 μmol/L probe and 6.45μL sterile distilled water are mixed, 5 μL DNA sample solution is added. As a negative control PCR reaction, an equal volume of the sterile distilled water is added instead of the DNA sample solution[*2]. If the 96-well reaction plate is used, the plate must be completely sealed[*3] using the sealing applicator. When using ABI7900HT detection system, the MicroAmp Optical Cover Compression Pad[*4] is placed on the reaction plate. Each DNA sample solution

is tested duplicate using the GM potato (F10, J3) and the potato positive control detection methods.

*1 TaqMan Gene Expression Master Mix

The solution prepared using this reagent has a high viscosity; therefore, mixing should be carefully performed. If the mixing is not properly performed, the PCR may fail. The PCR Master Mix should be mixed and centrifuged before use. Considering that the reaction solution is not easy to be mixed and centrifuged when the PCR Master Mix is contained, it is suggested to pipette all solution at the bottom of the plate.

*2 Non-Template Control (NTC)

The NTC is prepared by adding 5.0 μL water in place of the DNA sample solution.

*3 The 96-well plate, the adhesive film and the adhesive film applicator

Use MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.). When sealing with the reaction film, follow the manufacture's instruction.

*4 In the case of ABI PRISMTM 7900, use the MicroAmp Optical Cover Compression Pad, but do not use in the case of ABI PRISM™ 7500.

5.2. Setting the plate information

To start reaction, the plate information (sample position, types and probe characteristics) must be set. The sample ("NTC", Non-Template Control; "UNKN", DNA sample solution) is set according to the positions of the samples prepared. Also, with respect to the probe characteristics, "FAM" as a reporter and "TAMRA" as a quencher are used in F10, J3 and APRT detection tests. In addition, the passive reference is set to "ROX". The run mode is set to the 9600 emulation mode.

5.3. PCR amplification

The plate is set to the instrument, and then the reaction is started. The reaction condition is as follows. After pre-heating at 50°C for 2 min and 95°C at 10 min, the thermal cycles are carried out 45 cycles of 95°C for 15 sec and 60°C for 1 min. After making sure that the remaining time is zero when the reaction is terminated, and the results are analyzed.

6. Analysis and decision of result (**see Figure 1**)

The GM potato (F10, J3) and the potato positive control (APRT) are detected based on the confirmation of the exponential amplification curve in the amplification plot, the Ct (Cq) value and the clear exponential increase in the fluorescence (FAM) intensity through the multicomponent analysis. When the exponential amplification curve in the amplification plot is confirmed visually from both the GM potato and the positive control (APRT), the samples are suspected for the GM potato contamination.

F10 and J3 detections:

(4) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection and the Ct (Cq) values of all duplicate sample wells in both of the GM potato (F10, J3) detection and the APRT detection are less than 43 (STEP2 pattern1), the sample is contaminated with the GM potato.

(5) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection, but the Ct (Cq) values of all duplicate sample wells are not less than 43 (STEP2 pattern2) in both of the GM potato (F10, J3) detection and the APRT detection, the sample is not contaminated with the GM potato.

(6) If the Ct (Cq) values of both duplicate sample wells are less than 43 in the papaya positive control detection

and the Ct (Cq) values of both the GM potato (F10, J3) detection and the APRT detection are neither pattern1 nor pattern2 of STPE2, the DNAs are extracted and purified again from the ground sample, and the real-time PCR detection is further performed. If the result still shows positive when the second DNA sample prepared is used, the sample is not contaminated with the GM potato.

The pattern of the GM potato detection results

	positive control (APRT)	GM potato
DNA sample-1	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)

In addition, when the Ct (Cq) values of all wells are less than 43 in the potato positive control detection, the DNAs are extracted and purified again from the ground sample, and the real-time PCR detection is performed again. If the result can not be obtained using the second DNA sample preparation, the detection from this sample is concluded not testable.

Salmon (AquAdvantage) detection method

In this method, fresh salmon and salmon-food products are subjected for testing, and anion exchange resin type kit method (QIAGEN Inc. Genomic-tip 20 / G) or silica gel membrane type method (NIPPON GENE GM quicker 3) were used for DNA extraction and purification. The detection of GM salmon (AquAdvantage) is determined from duplicate qualitative real-time PCR tests per a sample using primers and probe set for AquAdvantage and salmon positive control detection. For AquAdvantage detection, primers and probe that are designed to detect the splicing region of growth hormone gene from North Pacific Ocean salmon (*Oncorhynchus tshawytscha*) are used. For positive control detection of salmon, primers and probe set that are designed to detect 18SrRNA gene sequence from salmon were used.

DNAs are extracted and purified in duplicate from a sample, and qualitative real-time PCR is carried out using the obtained DNA samples.

1. DNA extraction and purification from fresh and salmon-food products

Raw salmon and its processed foods were classified into the following three types of products, performing sample preparation prior to DNA extraction and purification according to each sample pretreatment protocol as follows.

① Raw salmon and its processed food (smoked salmon, canned (boiled, etc.), salmon flakes)
② Dried product (rice-sprinkled, such as Ochazuke)
③ Salmon eggs and its processed food

1.1. Sample pretreatment

1.1.1. Raw salmon and its processed food (smoked salmon, canned (boiled, etc.), salmon flakes)

Single package (For over-sized products, 120 g edible parts except the fat content are used) is tested, an equal weight distilled water is added, and the sample is ground using food processor. The obtained ground sample (1 g) is weighed into a polypropylene centrifuge tube (50 mL-capacity).

1.1.2. Dried product (rice-sprinkled, such as Ochazuke)

Single package (or 120 g) is tested and ground using food processor. The ground sample 0.5 g is weighed in a polypropylene centrifuge tube (50 mL capacity).

1.1.3. Salmon eggs and its processed food

Fifty grams of salmon eggs are tested. An equal weight fraction of distilled water is added, and the sample is ground using a food processor. The ground sample (1 g) is weighed into a polypropylene centrifuge tube (50 mL capacity).

1.2. DNA extraction and purification from salmon sample

For DNA extraction and purification, the pre-treated samples that are not buffer-absorbable (such as raw salmon, smoked-salmon, canned salmon, salmon flakes, etc.) should follow Method A. The pre-treated samples that are buffer-absorbable (such as dried salmon flakes, etc.) should follow Method B. The pre-treated samples that contain oil (such as salmon eggs, etc.) should follow "1.2.1.3. Ion exchange resin type DNA extraction and purification kit method".

1.2.1. DNA extraction and purification

1.2.1.1. Silica gel membrane type kit method (NIPPON GENE GM quicker 3) ---Method A

[for the pre-treated samples that are not buffer-absorbable (such as raw salmon, smoked-salmon, canned salmon, salmon flakes, etc.])

To samples that weighed in the polypropylene centrifuge tube, 1 mL GE1 buffer[*1], 10 μL RNaseA[*1] and 5 μL Proteinase K[*1] are added and mixed in a test tube by vortexing for 30 seconds so that no samples are remained, then incubate at 65°C for 30 minutes. During the incubation, tubes are mixed vigorously for 10 seconds twice per 10 minutes. Then, 200 μL GE2-P Buffer[*1] is added and mixed well. Then, samples are centrifuged at 4,000×g, 4°C for 10 minutes and 800 μL resulting supernatant is transferred into a 2-mL tube. Then, 600 μL GB3 Buffer[*1] is added and tubes are inverted 10-12 times. Then, tube is centrifuged for 15 minutes at over 12,000×g, 4°C and the supernatant is isolated. Then, 700 μL resulting supernatant is transferred to the spin column and the tube is centrifuged for 30 seconds at over 10,000×g, 4°C, and the supernatant is discarded. Likewise, the supernatants from the other tubes are transferred to the spin column and tubes are centrifuged for 1 minute at over 10,000×g, 4°C. Then, 600 μL GW buffer[*1] is placed to the column, the tube is centrifuged for 30 seconds at over 13,000×g, 4°C (the supernatant is discarded). Then, the spin column is transferred to a 1.5 mL tube, and 50 μL distilled water is placed directly onto the spin column and incubated for 3 minutes at room temperature, and then, the tubes are centrifuged for 1 minute at over 10,000×g to elute DNAs.

[*1] GE1 buffer, GE2-P buffer, GB3 buffer, GW buffer, RNaseA and ProteinaseK are used from the silica gel membrane type kit (NIPPON GENE GM quicker), or all buffers can be prepared according to the kit's instructions.

1.2.1.2 Silica gel membrane type kit method (NIPPON GENE GM quicker 3) --- Method B
(for absorptive sample; dry product (sprinkled, such as Ochazuke)

To samples that weighed to polypropylene centrifuge tube, 2.25 mL GE1 buffer[*1], 10 μL RNaseA[*1] and 5 μL Proteinase K[*1] are added and mixed in a test tube mixer for 30 seconds so that no sample are left remained and incubated at 65°C for 30 minutes. During incubation, tubes are mixed vigorously for 10 seconds two times per 10 minutes. Then, 250 μL GE2-P Buffer[*1] is added and test tubes are mixed well. Then, samples are centrifuged at 4,000×g, 4°C for 10 minutes and 800 μL resulting supernatant is transferred into a 2.0 mL tube. Then, 600 μL GB3 Buffer[*1] is added and tubes are inverted 10-12 times. Then, the tubes are centrifuged for 15 minutes at over 12,000×g, 4°C and the supernatant is isolated. Then, 700 μL resulting supernatant is transferred to the spin column and tubes are centrifuged for 30 seconds at over 10,000×g, 4°C, and supernatant is discarded. Likewise, the supernatant from the other tubes is transferred to the spin column and the tubes are centrifuged for 1 minute at over 10,000×g, 4°C. Then, 600 μL GW buffer[*1] is placed to the column, the tube is centrifuged for 30 seconds at over 13,000×g, 4°C, and the supernatant is discarded. Then, the spin columns are transferred to a 1.5 mL tube, and 50 μL distilled water is placed directly onto the spin column and incubated for 3 minutes at room temperature, and then, the tubes are centrifuged for 1 minute at over 10,000×g to elute DNA.

[*1] GE1 buffer, GE2-P buffer, GB3 buffer, GW buffer, RNaseA and ProteinaseK are used from the silica gel membrane type kit (NIPPON GENE GM quicker), or all buffers can be prepared according to the kit's instructions.

1.2.1.3. Ion exchange resin type DNA extraction and purification kit method (QIAGEN Genomic-tip 20/G) (for salmon eggs and its processed foods)

Into the ground sample that was weighed in a polypropylene centrifuge tube (50 mL capacity), 8 mL G2 buffer *[1], 10 μL RNaseA[*2] and 50 μL Proteinase K[*2] are added and mixed vigorously by vortex mixer to homogenous, and incubated for 1 hour at 50 °C. During incubation, tubes are mixed two to three times. Then, samples are centrifuged at over 3,000×g, 4 °C for 15 minutes. The resulting supernatant is transferred into a polypropylene centrifuge tube (15 mL capacity) and centrifuged at over 3,000×g, 4 °C for 15 minutes. And then, the resulting supernatant is transferred into QIAGEN Genomic-tip 20 /G that was equilibrated with 1 mL QBT buffer*[1]. Then, the column is washed using 2 mL QC buffer[*1] three times, and the column is transferred to a new centrifuge tube, 1 mL-QF buffer[*1], which was pre-warmed to 50°C, is loaded twice, and DNA was eluted. An equal volume of isopropanol is added to eluate DNAs and mixed solely. Then, transfer the DNA sample to a new 1.5 mL or 2.0 mL centrifuge tubes, and the tubes are centrifuged for 15 minutes at 10,000×g at room temperature, then the supernatant is discarded. One microliters of 70% ethanol is used to rinse the pelette. After centrifugation for 5 minutes at 10,000×g, 4°C, the supernatant is discarded, then the pellette is dried. Fifty microliters water, which was pre-warmed to 50°C, is added to dissolve the pellette. Concentration and purity of the extract DNA sample is estimated using a spectrophotometer.

[*1] G2 buffer, QBT buffer, QC buffer, and, QF buffer are used from the kit, or all buffers can be prepared according to the kit's instructions.
[*2] RNaseA and Proteinase K are used from the kit, or the other products with the equivalent activity can be used.

1.2.2. Detection of DNA purity in undiluted DNA sample solution and preparation and storage of DNA sample solution

An aliquote of undiluted DNA sample solution is diluted using sterile distilled water, and absorbance of resulting solution is measured at 260 and 280 nm absorbance (A260 and A280[*1], respectively). DNA concentration is measured at one A260 as 50 ng/μL DNA. If A260/A280 ratio is 1.7 to 2.0, DNA has been substantially purified[*2]. From the resulting DNA concentration, the DNA sample stock solution is prepared by diluting to 10 ng/μL using sterile distilled water, and the DNA sample solution. DNA sample solution is transfer to micro-centrifuge tube for each 25 μL, and stored frozen at less than -20°C. DNA sample solution is used immediately after thawing, the remaining solution is discarded without saving again. Incidentally, when the concentration of the DNA sample stock solution does not reach 10 ng/μL, then the DNA sample solution directly used.

[*1] A260 is consider the absorbance measurements for DNA concentration, A280 is considered the absorbance measurements for protein in the sample.
[*2] No further DNA purification is required when A260/A280 ratio is outside the range of 1.7-2.0.

2. Qualitative real-time PCR (ABI PRISM™ 7900 or 7500)

Aquadvantage is detected using 2 real-time PCR methods to detect AquAd and salmon endogenous gene. Each primer and probe is dissolved in sterile distilled water. Sequences of primers and probe are as follows.

Primers and probe for detecting AquAdvantage
AquAd F: 5’- TGC TGA TGC CTC TGA TAC CAC- -3’
AquAd R: 5’- ATG CCT CTA GTG CAA GTT CAG TC -3’
AquAd P: 5’-FAM- CAG TAG TAC AAC GTT GGC AGA TGT ATG AGA ACT -BHQ1-3’

Primers and probe for detecting endogenous gene
18S F: 5’- TGT GCC GCT AGA GGT GAA ATT -3’
18S R: 5’-GCA AAT GCT TTC GCT TTC G -3’
18S P: 5’-FAM- TTG GAC CGG CGC AAG ACG G-TAMRA-3’

2.1. Preparation of reaction solution for PCR

Total volume of PCR reaction solution is set at 25 μL/well and prepared as follows. After 12.5μL of TaqMan Gene Expression Master Mix [*1], 0.4 μL 50 μmol/L primer, 0.25 μL 10 μmol/L probe and 6.45 μL with sterile distilled water were mixed, 5 μL DNA sample solution was added. As a negative control PCR reaction, an equal amount of sterile distilled water is added instead of the DNA sample solution[*2]. If 96-well reaction plate is used, completely seal[*3] using sealing applicator. When using ABI7900HT detection system, place MicroAmp Optical Cover Compression Pad[*4] above the reaction plate. Each DNA sample solution is tested duplicate using AquAdvantage detection and salmon positive control detection methods.

* 1 TaqMan Gene Expression Master Mix or EagleTaq Master Mix with ROX

Solution prepared using this reagent has a high viscosity; therefore, mixing should be carefully performed. If mixing is not properly performed, PCR may fail. Universal PCR Master Mix should be mixed and centrifuge before use. Considering that the reaction solution is not easy to be mixed and centrifuged when Universal PCR Master Mix is contained, it is suggested to pipette all solution at the bottom of the plate.

* 2 Non-Template Control (NTC)

Upon addition of DNA sample solution, NTC is prepared by adding 2.5 μL of water in place of the DNA sample solution.

* 3 96-well plate, adhesive film and adhesive film applicator

Use MicroAmp Optical 96-Well Reaction Plate and the ABI PRISM Optical Adhesive Cover (Thermo Fisher Scientific, Inc.). When sealing the reaction film, refer to the manufacture’s instruction.

* 4 MicroAmp Optical Cover Compression Pad (the case of ABI PRISMTM 7900, Thermo Fisher Scientific, Inc.)

Do not use in the case of ABI PRISM™ 7500

2.2. Setting plate information

To start reaction, plate information (sample position, types and probe characteristics) must be set. The sample ("NTC": Non-Template Control, "UNKN": DNA sample solution) is set according to the positions of the samples prepared. Also, with respect to the probe characteristics, AquAd and 18S, use "FAM" as a reporter and "TAMRA" as a quencher. In addition, Passive Reference is set to "ROX". The run mode is set to 9600 emulation mode.

2.3. PCR amplification

Plate is set to the instrument, and the reaction is initiated. The reaction condition is as follows. After pre-heating at 50°C for 2 minutes and 95°C at 10 minutes, the thermal cycles are carried out 45 cycles of 95°C for 15 sec and 60°C for 1 min. After making sure that the remaining time is zero when the reaction was terminated, and analysis of the measurement is performed.

3. Analysis and decision of result (**see Figure 1**)

The detections of GM salmon (AquAdvantage) and salmon positive control are determined based on the confirmation of the exponential amplification curve in the amplification plot and the Ct (Cq) value (clear exponential increasing of the fluorescence intensity from subject dye [FAM]) on the multicomponent. When the exponential amplification curve on the amplification plot is confirmed visually from both of the GM salmon and salmon positive control, the samples are suspected the GM salmon (AquAdvantage)-positive.

Decision of AquAdvantage

(1) If the endogenous gene detections are less than 43, and Ct (Cq) values of both duplicate sample wells on the GM salmon detection at Ct (Cq) values of all duplicate sample wells are at less than 43 (STEP2 pattern1), the sample is AquAdvantage-positive.

(2) If the endogenous gene detections are less than 43, and Ct (Cq) values of both duplicate sample wells on the GM salmon detection at Ct (Cq) values of all duplicate sample wells are not at less than 43 (STEP2 pattern1), the sample is AquAdvantage-negative.

(3) If the results from samples are not matched, the DNA samples are extracted and purified again from the ground sample, and the real-time PCR is repeated. If the second DNA sample solution are not reached a conclusion, the sample is AquAdvantage-negative.

The pattern of AquAdvantage-positive result

	positive control (18S)	AquAdvantage
DNA sample-1	(+/+)	(+/+)
DNA sample-2	(+/+)	(+/+)

If endogenous gene detection failed, the DNA are extracted and purified again from the ground sample. If the second DNA samples failed to detect at Ct (Cq) values at less than 43, then the sample is concluded impossible to test.

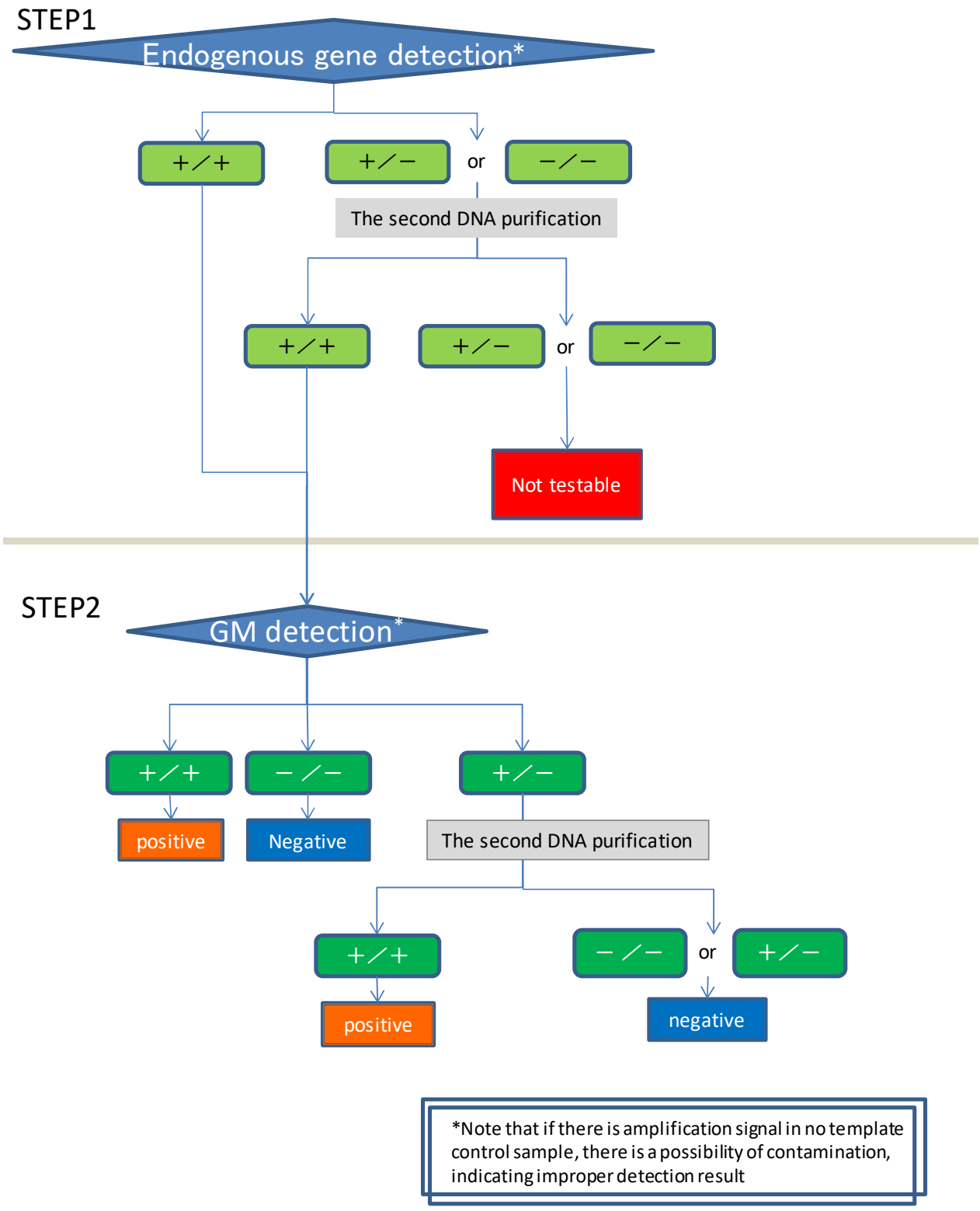


Figure 1. Detection scheme

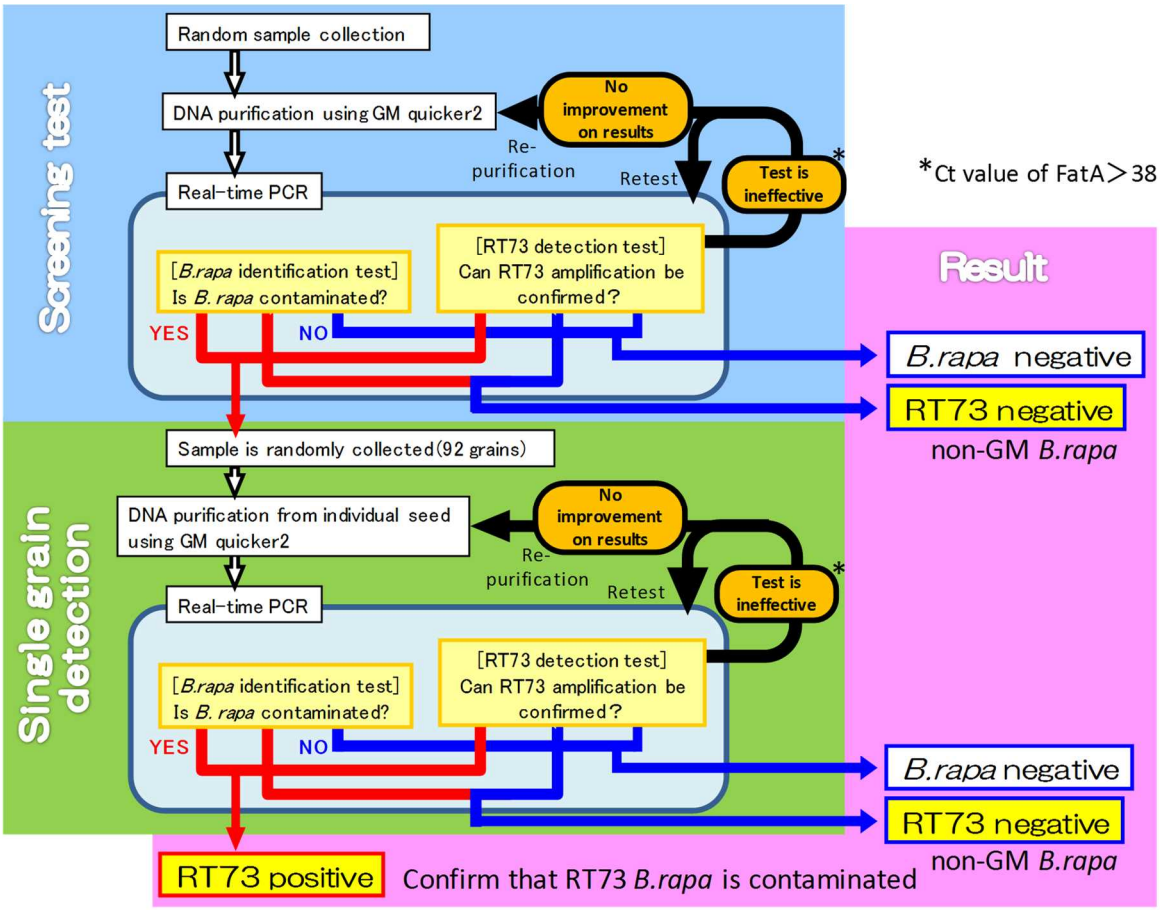
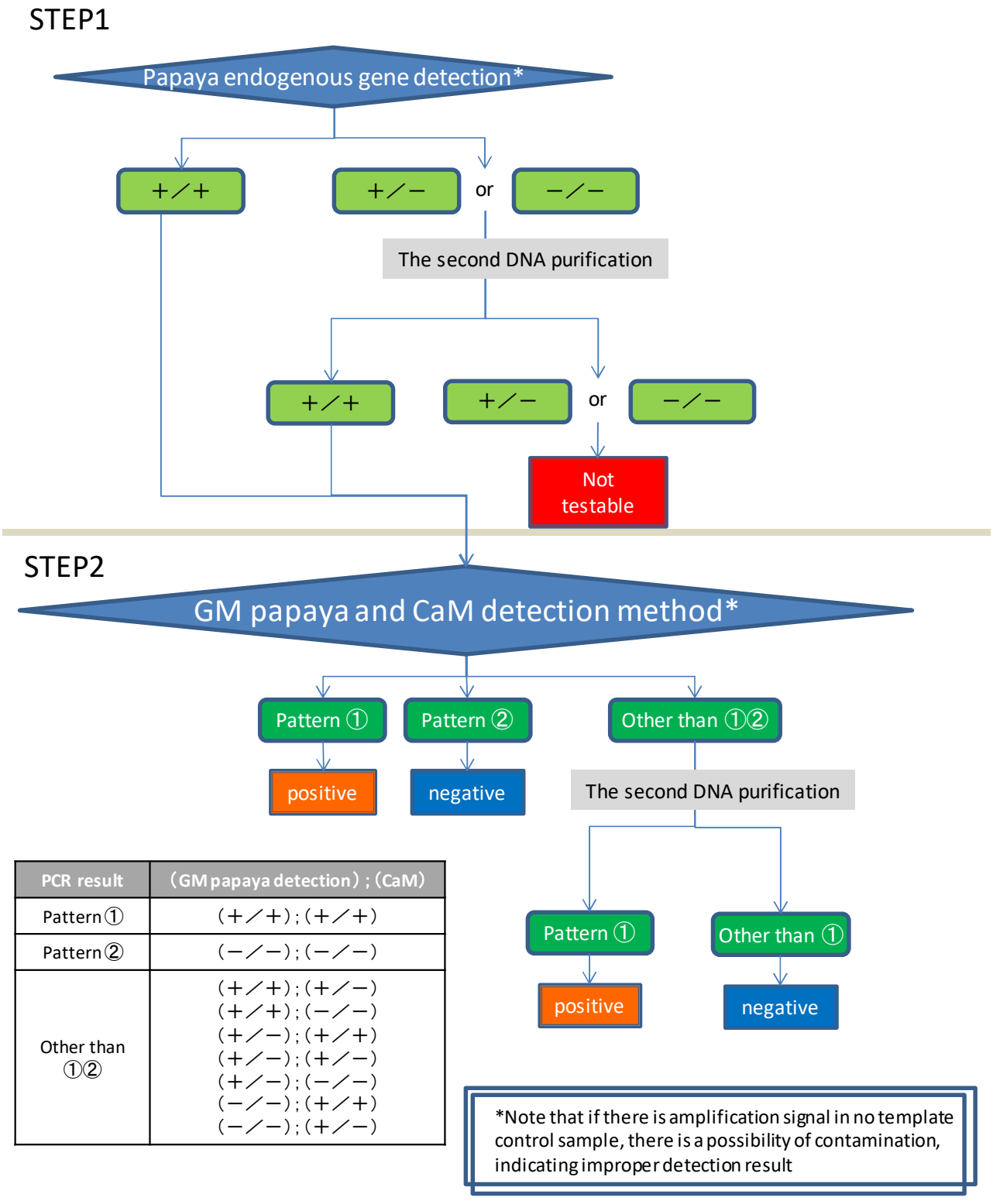


Figure 2. RT73 *B.rapa* detection scheme



PCR result	(GM papaya detection) ; (CaM)
Pattern①	(＋／＋) ; (＋／＋)
Pattern②	(－／－) ; (－／－)
Other than ①②	(＋／＋) ; (＋／－) (＋／＋) ; (－／－) (＋／－) ; (＋／＋) (＋／－) ; (＋／－) (＋／－) ; (－／－) (－／－) ; (＋／＋) (－／－) ; (＋／－)

Figure 3. PRSV-YK, PRSV-SC, HN detection scheme

III. Equivalence check for the detection method

1. DNA extraction and purification method

"II. Detection methods" section shows a representative. So far, DNeasy Plant Mini (Maxi) kit, Genomic tip-20, -50 and -100, GM quicker 3, etc. are commercially available for the DNA extraction. The choice of the samples differs among the research labs, and the sample matrices differ among the samples substantially. Therefore, the optimized DNA extraction and purification methods are selected considering the type of the tests and the samples. For the DNA extraction and purification method, it is necessary to obtain the DNA samples at the level of the purity and the quantity necessary for the testing with good reproducibility. For example, new DNA extraction and purification method is tested in six consecutive runs during three different days and confirmed equivalent as the existing methods from the real time PCR results. The results from the endogenous gene detection (the positive control test) are confirmed such that there is no difference in the Ct values (The difference remains within the Ct value <1).

2. Real-time PCR instrument

The other instruments not listed in the “II. Detection methods” section can be used (other than the ABI PRISM 7900 or the LightCycler 96/480). The equivalence in the instrument performance is confirmed by considering the sensitivity, the repeatability, the difference among wells, the amplification efficiency (especially when quantifying), etc. For example, having a positive control plasmid (e.g., for the rice testing) ready, the diluted solutions are prepared at the concentration slightly higher than the detection limit (the lowest concentration detected at all 10 tests) using the current instrument model (ABI PRISM 7900, etc.). Using this equivalent solution, the instrument model of the interest is tested in parallel, and the positive signal must be detected in all three tests during different days. Make sure that there is no difference among the 96 wells (The difference remains within the Ct value <1).

3. Master Mix

Using the instruments described in "II. Detection methods" section or the equivalent instrument confirmed, the endogenous gene detection method is tested three or more times using the crop sample for detection (if the crop sample is not available, low processed product etc. is allowed). As a result, no large difference between the Ct values and the endpoints as described in "II. Detection methods" section is confirmed. Also, no large difference in the Ct values and the end points is confirmed in the recombinant gene detection method part by testing three or more times using undiluted commercially available positive control plasmid (The difference remains within the Ct value <1).