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## Korea, Republic of

### Biotechnology

## Korean Testing Methods for GM Corn, Soybeans, and Potatoes

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**Report Highlights:**

The Korea Food & Drug Administration (KFDA) provided a translation of Korea's official testing methods for biotech corn, soybeans, and potatoes. This report also provides recommended information that foreign laboratories include on their testing certificates for products exported to Korea.

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Includes PSD Changes: No  
Includes Trade Matrix: No  
Unscheduled Report  
Seoul [KS1]  
[KS]

Currently the Korea Food & Drug Administration (KFDA) has accredited four Korean domestic laboratories for GMO testing. No laboratories in foreign countries have been accredited so far. Beginning December 1, 2005, KFDA required accredited domestic laboratories to submit testing certificates that contain information on the 17 items listed below. KFDA would like foreign laboratories to complete the tests when issuing certificates for products destined for Korea, even if the laboratories are not accredited. To facilitate compliance KFDA has provided a translation of its testing methods and requirements.

The accreditation of local laboratories for GMO testing does not change the terms of enforcement of GMO labeling requirements. KFDA requires either full IP documents, a government issued certificate, or a notarized self-certification (for US origin products only) when determining the exemption from GMO labeling. Importers and exporters can submit testing certificates issued by either Korean or foreign laboratories (government laboratories or private laboratories) for products that do not contain foreign protein or DNA. However, if a product tests positive for a GMO component during KFDA's random testing, it will require a label stating that the product is GMO regardless of what is stated on the original testing certificates. Testing certificates issued by accredited laboratories do not guarantee that products are exempt from random inspection.

KFDA believes accrediting local laboratories for GMO testing will improve the credibility of the testing results but will not replace KFDA's random testing.

The 17 items that will be included on the testing certificates are as follows:

1. Sample name (Korean, English)
2. Manufacturer and importer (or client) name
3. Production date and lot number of the product
4. Sample weight submitted (kg)
5. Sample packing appearance
6. Sample description
7. Dates test started and completed
8. DNA extraction reagent or DNA extraction kit (e.g. Qiagen kit, CTAB method, etc.)
9. Sample weight (g) used per DNA extraction and repetition number of DNA extraction
10. Concentration (ng/ul) and purity ( $A_{260}/A_{280}$ ,  $A_{260}/A_{230}$ ) of Extracted DNA and Elution volume (ul)
11. Accredited method or reference used for PCR (e.g. kit or primer name)
12. Target DNA region for PCR (e.g. Lectin, P35S, etc.)
13. Repetition number for PCR
14. Limit of Detection (LOD)
15. Concentration (ng/ul) and Quantity (ng) of Extracted DNA used per PCR
16. Electrophoresis picture images of PCR products (positive and negative controls should be included)
17. Final test result (Detected, Not detected, Indeterminate)

Note: The translation below is provided by KFDA. However, if there is any discrepancy between the English translation and the original Korean text, the Korean texts shall prevail.

## Korean Food Code

### Chapter 7. Testing Methods in General

#### 23. Testing methods of GM foods

Testing methods of genetically modified foods involve recombinant genes and foreign proteins derived from such genes, and refer to polymerase chain reaction (PCR) analysis for recombinant genes, and immunoassay based on antigen-antibody reaction for foreign proteins.

For agricultural products such as grains and pulses and slightly processed agricultural products made by simply crushing agricultural products, both analytical methods for recombinant genes and foreign proteins may be used while the analytical methods for recombinant genes, not analytical methods for proteins, are used for processed foods due to denaturing, decomposition, of proteins during manufacturing/processing. For the analysis of foreign proteins, commercial test kits using enzyme-linked immunosorbent assay (ELISA) techniques or lateral flow strips may be used.

The qualitative analysis of recombinant genes uses standard PCR equipment while the quantitative analysis of the genes uses real-time PCR equipment.

However, both qualitative and quantitative analytical methods are applicable to agricultural products and their merely ground products while only the qualitative analysis is applied to processed foods until the quantitative method is developed and established.

#### 1) Pre-treatment of samples

##### (1) Washing and grinding of grains/pulses

Take at least 3,000 kernels or more than 1 kg of a grain or pulse sample and homogeneously grind them using a grinder. Such ground powder is used for analysis. If the sample contains chemicals or other extraneous materials that may affect test results, put the sample in 1% SDS (Sodium Dodecyl Sulfate) solution and stir it well until bubbles are sufficiently formed. After washing, remove the solution and rinse the sample with distilled water more than 10 times until such bubbles disappear. Dry the sample and use it for analysis.

##### (2) Pre-treatment of processed foods with high water content

For processed foods with high water content, such as soybean milk, homogeneously grind solid materials obtained from centrifugation at 8,000xg for 15 minutes or from overnight drying at 55 °C or evaporation of water using a freeze dryer and use such ground powder for analysis.

##### (3) Pre-treatment of processed foods with high sugar content

For processed foods with high sugar content, such as sweet stuffs coated with syrup, grind them with a grinder, add distilled water to dissolve the sugar, perform centrifugation, and take the solid portion. Repeat this procedure until sugar is sufficiently removed. Use the resultant solid material for analysis.

##### (4) Pre-treatment of processed foods with high absorptive property

For processed foods with high absorptive property, add appropriate quantity of sterilized distilled water, homogenize them with a grinder, perform centrifugation at 8,000xg for 15 minutes at room temperature, and take the solid material for analysis.

### **2) Weighing of samples and prevention of contamination**

To weigh samples for extraction of DNA, clean a precision scale and its neighboring area with 70% ethanol to prevent contamination. Then, place the plastic wrap generally used for food packaging over the scale and conduct weighing of samples in tubes to be used in extraction of DNA. If possible, use disposable materials for weighing. Otherwise, use different ones for different samples. After weighing, clean the tube mouth and close the tube.

### **3) Extraction and purification of DNA**

Extraction and purification of DNA can be conducted by the CTAB method (extraction and purification of DNA using a mixture of a surfactant, Cetyl Trimethyl Ammonium Bromide (CTAB), and phenol/chloroform) or the method using the DNA extraction kit in silica gel membrane type, silica-based resin type, or magnet-adsorptive bead type.

The CTAB method is applicable to various areas, leaves virtually no PCR inhibitors, and provides high-purity DNA. However, currently marketed DNA extraction kits have limited application areas, so their applicability to a specific type of processed foods has to be separately evaluated.

With use of such methods, DNA can be extracted from soybeans, corns, potatoes, or their processed products, and purified for PCR analysis. Conduct the DNA extraction process two times and perform PCR analysis for each DNA extract.

Distilled water in this test method means high-purity water of 17 MO/cm produced through reverse osmosis membrane or others, unless otherwise specified. Such distilled water should not be contaminated with DNA or DNase.

#### **(1) CTAB method**

Place 2g of the ground sample in a polypropylene tube (50ml), add 15ml of CTAB buffer, and homogenize the mixture using a vortex mixer. Add 30ml of CTAB buffer and leave it at 55 °C for 30 minutes. Take 600µl of the mixture sufficiently mixed and homogenized into 1.5ml tube, add 500µl of phenol/chloroform mixture into the tube, mix the solution well, and centrifuge it at 7,500xg for 15 minutes at room temperature. Transfer the supernatant to a new tube (1.5ml), add 500µl of chloroform/isoamyl alcohol mixture, mix it well using a mixer, and centrifuge it at 7,500xg for 15 minutes at room temperature. Transfer the supernatant to a new tube (1.5ml), add the same quantity of isopropyl alcohol, mix the solution by overturning the tube (about 10 times), and centrifuge it at 7,500xg for 10 minutes at room temperature. Discard the clear supernatant. Gently add 500µl of 70% ethanol along the wall of the tube to the pellet, centrifuge it at 7,500xg for 1 minute at room temperature, and suck out as much of the supernatant using a micropipette as possible without touching the pellet. Then, dry the pellet, being careful not to dry it completely. Add 50µl of TE buffer (pH 8.0), mix the solution well, and leave it at room temperature for 15 minutes for complete dissolution while overturning it occasionally. (If it is not dissolved well, leave it at 4 °C for 12 to 24 hours for complete dissolution.) To purify such DNA extract, add 5µl of RNase A (10mg/ml) and leave the mixture at 37 °C for 30 minutes. After adding 200µl of CTAB buffer, add 250µl of chloroform-isoamyl alcohol mixture, mix the solution using a mixer, centrifuge it at 7,500xg for 15 minutes at room temperature, and transfer the supernatant to a new 1.5ml tube. During this process, be careful not to disturb the medium layer. Add 200µl of isopropyl alcohol, mix the solution by overturning the tube about 10 times, and centrifuge it at 7,500xg for 10 minutes at room temperature. Discard the supernatant using a micropipette without touching the pellet. Add 200µl of 70% ethanol along the wall of the tube to the pellet and then, remove the supernatant using a

micropipette. Centrifuge it at 7,500xg for 1 minute at room temperature, suck out as much ethanol as possible using a micropipette, and dry the pellet, being careful not to dry it completely. Add 50 µl of sterilized distilled water or TE buffer (pH 8.0) to the dried pellet and leave it at room temperature for 15 minutes. During this process, overturn the tube occasionally to dissolve it completely. Use the solution as the DNA sample stock solution. (If it is not dissolved well, leave it at 4 °C for 12 to 24 hours for complete dissolution.)

[Preparation of solutions]

① CTAB buffer

Place 8 ml of 0.5M EDTA (Ethylene Diamine Tetra Acetic Acid) solution (pH 8.0), 20ml of 1M Tris/HCl buffer (pH 8.0), and 56ml of 5M salt solution in a beaker, add enough distilled water to bring the volume to approximately 150ml, add 4g of cetyl trimethyl ammonium bromide (CTAB) while stirring, and dissolve it completely. Add enough distilled water to bring the total volume to 200ml and autoclave the solution at 121 °C for 15 minutes.

② Phenol/chloroform mixture

Mix phenol saturated with 1M Tris/HCl solution (pH 8.0), chloroform, and isoamyl alcohol at 25:24:1 (v/v/v).

③ Chloroform/isoamyl alcohol mixture

Mix chloroform and isoamyl alcohol at 24:1 (v/v).

④ TE buffer (pH 8.0)

Prepare a solution having final concentrations of 10 mM Tris/HCl (pH 8.0) and 1 mM EDTA (pH 8.0) with sterilized distilled water.

(2) Silica-gel membrane-type kit method

Extract DNA using QIAGEN Plant Maxi kit or other equivalent kits according to the manufacturer's instructions.

① Soybeans or processed soybean products

When QIAGEN Plant Maxi kit is used, place 1g of uniformly ground sample in a polypropylene tube (50ml), add 10ml of AP1 buffer previously warmed at 65 °C and 20µl of RNase A (100 mg/ml), mix them well using a mixer, and leave the mixture at 65 °C for 1 hour. During this process, use a mixer for 10 seconds at the 15-minute intervals for complete mixture. After 1-hour reaction, centrifuge it at 3,000xg for 10 minutes at room temperature and take the supernatant (7ml) into a new 50ml tube, being careful not to touch the pellet and the membrane formed. Add 2.5ml of AP2 buffer, leave the mixture in ice for 15 minutes, and centrifuge it at 3,000xg for 35 minutes at room temperature. Then, transfer the clear supernatant (8ml) to a QIA Shredder Spin Column, and perform centrifugation at 3,000xg for 5 minutes at room temperature using a swing rotor. Transfer the elute (7.5ml) into a new 50ml tube, mix it well for 10 seconds, and place the solution (6.8ml) in a new 50ml tube. Add 1.5 times the elute volume of AP3 buffer/ethanol mixture (10.2 ml), mix it well for 10 seconds, apply it to a DNeasy Maxi Spin Column, and perform centrifuge at 3,000xg for 5 minutes at room temperature using a swing rotor to have DNA attached to the column. Discard the elute. Apply 12ml of AW buffer to the column, perform centrifugation at 3,000xg for 15 minutes at room temperature, and conduct cleaning. Transfer the column into a new 50ml tube, add 1ml of sterilized distilled water previously warmed at 65 °C, leave it at room temperature for 5 minutes, and perform centrifugation at 3,000xg for 10 minutes at room temperature to elute DNA. Take the elute into 2ml tube, add the same quantity of isopropyl alcohol, overturn it 10 times, and leave it at room temperature for 5 minutes. Perform centrifugation at 12,000xg for 15 minutes at 4 °C and remove the supernatant using a micropipette, being careful not to touch the pellet. Add 500µl of 70% ethanol and gently dissolve the pellet attached to the wall of the tube. Perform centrifugation at 12,000xg for 3 minutes at 4 °C, remove the remaining ethanol using a micropipette, and dry the precipitate.

Add 50µl of sterilized distilled water or TE buffer (pH 8.0) to the dried precipitate and leave it at 4 °C for 12 to 24 hours for complete dissolution. Use this as the DNA sample stock solution.

② Corns or processed corn products

When QIAGEN Plant Maxi kit is used, place 1g of uniformly ground sample in a polypropylene tube (50ml), add 5ml of AP1 buffer previously warmed at 65 °C and 10µl of RNase A (100mg/ml), mix them well using a mixer, and leave the mixture at 65 °C for 1 hour. During this process, use a mixer for 10 seconds at the 15-minute intervals for complete mixture. Add 1.8ml of AP2 buffer, leave the mixture on ice for 15 minutes, and centrifuge it at 3,000xg for 15 minutes at room temperature. Then, transfer the clear supernatant (4.2ml) to a QIA Shredder Spin Column, and perform centrifugation at 3,000xg for 5 minutes at room temperature using a swing rotor. Transfer the elute (4ml) into a new 50ml tube, mix it well for 10 seconds, and place the solution (3.4ml) in a new 50ml tube. Add 1.5 times the elute volume of AP3 buffer/ethanol mixture (5.1ml), mix it well for 10 seconds, apply it to a DNeasy Maxi Spin Column, and perform centrifuge at 3,000xg for 5 minutes at room temperature using a swing rotor to have DNA attached to the column. Discard the elute. Apply 12ml of AW buffer to the column, perform centrifugation at 3,000xg for 15 minutes at room temperature, and conduct cleaning. Transfer the column into a new 50ml tube, add 1ml of sterilized distilled water previously warmed at 65 °C, leave it at room temperature for 5 minutes, and perform centrifugation at 3,000xg for 10 minutes at room temperature to elute DNA. Take the elute into 2ml tube, add the same quantity of isopropyl alcohol, overturn it 10 times, and leave it at room temperature for 5 minutes. Perform centrifugation at 12,000xg for 15 minutes at 4 °C and remove the supernatant using a micropipette, being careful not to touch the pellet. Add 500µl of 70% ethanol and gently dissolve the pellet attached to the wall of the tube. Perform centrifugation at 12,000xg for 3 minutes at 4 °C, remove the remaining ethanol using a micropipette, and dry the precipitate. Add 50µl of sterilized distilled water or TE buffer (pH 8.0) to the dried precipitate and leave it at 4 °C for 12 to 24 hours for complete dissolution. Use this as the DNA sample stock solution.

③ Potatoes and processed potato products

When QIAGEN DNeasy Plant Mini kit is used, place 200mg of uniformly ground sample in a centrifuge tube (15ml), add 1.5ml of AP1 buffer previously warmed at 65 °C and 10µl of RNase A (100mg/ml), mix it well using a mixer, and leave the mixture at 65 °C for 15 minutes. During this process, use a mixer for 10 seconds at the 5-minute intervals for complete mixture. Add 400 µl of AP2 buffer, leave the mixture in ice for 5 minutes, and centrifuge it at 10,000xg for 5 minutes at room temperature. Then, transfer the clear supernatant to another centrifuge tube, add 500µl of the supernatant to a QIA Shredder Spin Column, and perform centrifugation at 10,000xg for 2 minutes at room temperature. Transfer the elute into a new tube. Repeat this process for the remaining supernatant. Transfer 2ml of the elute to two 2ml tubes, add 1.5 times the elute volume of AP3 buffer/ethanol mixture, and mix them well for 10 seconds. Take 500 µl of the mixture into a DNeasy Mini Spin Column and perform centrifuge at 10,000xg for 1 minute at room temperature to have DNA attached to the column. Discard the elute. Apply 500µl of AW buffer to the column, perform centrifugation at 10,000xg for 1 minute at room temperature for cleaning. Repeat this process after addition of 500 µl of AW buffer. To dry the column, perform centrifugation at 10,000xg for 15 minutes at room temperature and transfer to a new 1.5ml tube. Add 50 µl of sterilized distilled water previously warmed at 65 °C, leave it at room temperature for 5 minutes, and perform centrifugation at 10,000xg for 1 minute at room temperature to elute DNA. Once again, add 50 µl of sterilized distilled water previously warmed at 65 °C and repeat this process. Use this as the DNA sample stock solution.

**Note**

AP1 buffer, AP2 buffer, and RNase A used in QIAGEN Plant Maxi kit and QIAGEN DNeasy Plant Mini kit for the silica-gel membrane-type kit method for extraction and purification of DNA are separately sold.

**4) Determination of the purity and concentration of DNA in DNA sample stock solution and its concentration**

Appropriately dilute the DNA sample stock solution with TE buffer (pH 8.0) and determine the absorbance (A) of such dilutions at 260 nm using a spectrophotometer. Use the value of 1 as 50ng/μl DNA. Meanwhile, measure the absorbance at 230 nm, 260 nm, and 280 nm to determine the purity of DNA extract. If the ratios of  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  are within the range of 1.7 to 2.0, the DNA is fully purified and suitable for PCR. However, such purity criteria cannot be applicable to processed foods. If the ratio of  $A_{260}/A_{280}$  is low and there may be contamination of protein impurities, treat the sample with protease and recover DNA. If the ratio of  $A_{260}/A_{230}$  is low, treat the sample with amylase and recover DNA for use in PCR.

Based on the determined DNA concentration of the DNA sample stock solution, dilute the DNA stock solution with TE buffer (pH 8.0) or sterilized distilled water to a concentration required for subsequent PCR reaction, mix it well, dispense the solution in 20 μl portions into 0.5ml tubes, and store them at below -20 °C for use as the DNA sample solution. Use the DNA sample solution for PCR reaction immediately after melting at room temperature and mixing it well. Any remaining sample solution after PCR reaction should not be used and should be discarded. Unless the concentration of the DNA sample stock solution reaches that required for PCR reaction, conduct extraction again. If the concentration is lower than that required for PCR even after re-extraction, use it as the DNA sample solution without dilution or conduct the following concentration process.

For concentration of DNA sample stock solution, add 1/10 times the DNA solution of 3M sodium acetate (pH 5.2) (1/5 times the DNA solution of 10M ammonium acetate), add 2 times the solution of cold ethanol, and perform centrifugation at 12,000xg for 15 minutes. (If isopropyl alcohol is used, add 1 time the solution and perform centrifugation at room temperature.) Discard the supernatant, clean the pellet with 70% ethanol to remove salts, dissolve it with appropriate volume of TE buffer (pH 8.0) or sterilized distilled water, and use the solution.

If the initial DNA sample stock solution is a small quantity, add sterilized distilled water to make 300 - 400μl and perform concentration.

**5) Qualitative analysis method**

Qualitative analysis is conducted with use of general PCR method. DNA extract is used as template DNA and amplified by use of primers to detect the amplified DNA by electrophoresis.

"In-house" primers or GMO detection kit for PCR reaction to detect GM foods can be used. As a very small amount of template DNA is amplified in PCR reaction, it is very important to avoid contamination of DNA other than target DNA (particularly PCR byproducts). Therefore, careful attention should be paid to devices, instruments, reagents, and environmental conditions. Further, DNase, secreted from the human skin surface, should be prevented from entering the PCR reaction mixture because it decomposes DNA. Therefore, disposable tubes and tips should be autoclaved at 121 °C for more than 20 minutes prior to use. All kinds of solutions to be used in preparation of various test solutions, except those vulnerable to heat, should be autoclaved at 121 °C for more than 15 minutes prior to use.

Contamination of sterilized distilled water or TE buffer (pH 8.0) with DNase will trigger the negative effects. Therefore, it is recommended that each technician prepare them for immediate use as necessary. When handling DNA, use an isolated space, such as clean bench. In addition, clean the workstation with 70% ethanol and wear non-powdered rubber gloves.

(1) Preparation of primers

① How to use "in-house" primers

Primers for test of GM soybeans, corns, or potatoes can be synthesized according to the method published in the AOAC Journal as shown in Table 1. Nippon Gene (Japan) makes and sells such primers (Table 2). Primers that will produce the equivalent results can be designed, prepared, and used.

② How to use commercial GMO detection kit

Commercial kits for test of GM soybeans, corns, or potatoes can be used. Such kits should have the performance equivalent to the method published in the AOAC Journal (see Table 1). In addition, primers that generate more than 200 bp of PCR byproducts are not suitable for analysis of processed foods.

Table 1. Primers and probes used in the method published in the AOAC journal.

Purpose	Products	Primers/ Probes	Base Sequences
Internal standard genes	Soybeans	Le1n02-5' Le1n02-3' Le1-Taq	5'-GCC CTC TAC TCC ACC CCC A-3' 5'-GCC CAT CTG CAA GCC TTT TT-3' 5'-FAM-AGC TTC GCC GCT TCC TTC AAC TTC AC-TAMRA-3'
	Corns	SSIIb 1-5' SSIIb 1-3' SSIIb-Taq	5'-CTC CCA ATC CTT TGA CAT CTG C-3' 5'-TCG ATT TCT CTC TTG GTG ACA GG-3' 5'-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3'
Screening	CMV p35S	P35S 1-5' P35S 1-3' P35S-Taq	5'-ATT GAT GTG ATA TCT CCA CTG ACG T-3' 5'-CCT CTC CAA ATG AAA TGA ACT TCC T-3' 5'-FAM-CCC ACT ATC CTT CGC AAG ACC CTT CCT-TAMRA-3'
	tNOS	NOS ter 2-5' NOS ter 2-3' NOS-Taq	5'-GTC TTG CGA TGA TTA TCA TAT AAT TTC TG-3' 5'-CGC TAT ATT TTG TTT TCT ATC GCG T-3' 5'-FAM-AGA TGG GTT TTT ATG ATT AGA GTC CCG CAA-TAMRA-3'
Structural genes	[Soybeans] RRS	RRS 01-5' RRS 01-3' RRS-Taq	5'-CCT TTA GGA TTT CAG CAT CAG TGG-3' 5'-GAC TTG TCG CCG GGA ATG-3' 5'-FAM-CGC AAC CGC CCG CAA ATC C-TAMRA-3'
	[Corns] Bt176	Bt176 2-5' Bt176 2-3' Bt176-Taq	5'-TGT TCA CCA GCA GCA ACC AG-3' 5'-ACT CCA CTT TGT GCA GAA CAG ATC T-3' 5'-FAM-CCG ACG TGA CCG ACT ACC ACA TCG A-TAMRA-3'
	Bt11	Bt11 3-5' Bt11 3-3' Bt11-Taq	5'-AAA AGA CCA CAA CAA GCC GC-3' 5'-CAA TGC GTT CTC CAC CAA GTA CT-3' 5'-FAM-CGA CCA TGG ACA ACA ACC CAA ACA TCA-TAMRA-3'
	GA21	GA21 3-5'	5'-GAA GCC TCG GCA ACG TCA-3'

		GA21 3-3'	5'-ATC CGG TTG GAA AGC GAC TT-3'
		GA21-Taq	5'-FAM-AAG GAT CCG GTG CAT GGC CG - TAMRA-3'
	T25	T25 1-5'	5'-GCC AGT TAG GCC AGT TAC CCA-3'
		T25 1-3'	5'-TGA GCG AAA CCC TAT AAG AAC CCT-3'
		T25-Taq	5'-FAM-TGC AGG CAT GCC CGC TGA AAT C- TAMRA-3'
	MON810	M810 2-5'	5'-GAT GCC TTC TCC CTA GTG TTG A-3'
		M810 2-3'	5'-GGA TGC ACT CGT TGA TGT TTG-3'
		M810-Taq	5'-FAM-AGA TAC CAA GCG GCC ATG GAC AAC AA-TAMRA-3'

Table 2. Primers prepared and sold by Nippon Gene (Japan)

Target Genes	Primer pairs	Amp. size	Remarks
[Soybeans]			
Internal standard genes	Le1n02-5' Le1n02-3'	118bp	<i>Le1</i> /sense <i>Le1</i> /antisense
Recombinant genes (transcription initiation)	P35S1-5' P35S1-3'	101bp	P35S/sense P35S-pro/antisense
Recombinant genes (transcription termination)	NOS ter 2-5' NOS ter 2-3'	151bp	tNOS/sense tNOS/antisense
Recombinant genes (structural genes)	RRS 01-5' RRS 01-3'	121bp	CTP4 from <i>P. hybrida</i> /sense <i>EPSPS</i> /antisense
[Corns]			
Internal standard genes	SSIIb 1-5' SSIIb 1-3'	151bp	<i>zSSIIb</i> /sense <i>zSSIIb</i> /antisense
Internal standard genes	SSIIb 3-5' SSIIb 3-3'	114bp	<i>zSSIIb</i> /sense <i>zSSIIb</i> /antisense
Recombinant genes (transcription initiation)	P35S 1-5' P35S 1-3'	101bp	P35S/sense P35S/antisense
Recombinant genes (transcription termination)	NOS ter 2-5' NOS ter 2-3'	151bp	tNOS/sense tNOS/antisense
Recombinant genes (Bt176 detection)	Bt176 2-5' Bt176 2-3'	100bp	<i>cryIA(b)</i> /sense PEPC#9 intron /antisense
Recombinant genes (Bt11 detection)	Bt11 3-5' Bt11 3-3'	127bp	<i>adh1-1S</i> /sense <i>cryIA(b)</i> /antisense
Recombinant genes (GA21 detection)	GA21 3-5' GA21 3-3'	133bp	OTP/sense <i>m-epsps</i> /antisense
Recombinant genes (T25 detection)	T25 1-5' T25 1-3'	149bp	<i>pat</i> /sense t35S/antisense
Recombinant genes (M810 detection)	M810 2-5' M810 2-3'	113bp	<i>hsp70</i> /sense <i>cryIA(b)</i> /antisense
Recombinant genes (CBH351 1st detection)	CaM03-5' CBH02-3'	170bp	CaM03/sense CBH02/antisense
Recombinant genes (CBH351 2nd confirmation)	Cry9C-5' 35Ster-3'	171bp	<i>cry9C</i> /sense t35S/antisense
[Potatoes]			
Internal standard genes	Pss 01n-5' Pss 01n-3'	216bp	<i>S. tuberosum</i> sucrose synthase/sense <i>S. tuberosum</i> sucrose synthase/antisense
Internal standard genes	UGPase01-5' UGPase01-5'	111bp	UGPase/sense UGPase/antisense

Recombinant genes (New Leaf Plus 1st detection)	p-FMV02-5' PLRV01-3'	234bp	p-FMV/sense PLRV/antisense
Recombinant genes (New Leaf Plus 2nd confirmation)	PLRV-rep1-5' PLRV-rep1-3'	172bp	PLRV/sense PLRV/antisense
Recombinant genes (New Leaf Y 1st detection)	p-FMV05-5' PVY02-3'	225bp	p-FMV/sense PVY/antisense
Recombinant genes (New Leaf Y 2nd confirmation)	pVY01-5' pVY01-3'	161bp	PVY/sense PVY/antisense

## (2) Preparation of reagents for PCR

Taq DNA polymerase (or polymerase producing an equivalent result) and other reagents should be prepared for PCR reaction according to the following procedures. To confirm the successful amplification of gene from the template (DNA extract), subject a mixture including a positive control primer pair (to detect the internal standard gene) to PCR reaction for each DNA sample solution.

### ① Preparation of standard DNA solution

Use the DNA solutions extracted from 0% and 2% standard sample (products manufactured by IRMM and sold by Fluka or other equivalent products) as the positive control. However, if PCR is conducted using primers from the method published in the AOAC Journal (Table 1), the positive-control plasmid prepared and sold by Nippon Gene can be used instead of DNA solution extracted from 2% standard sample.

### ② Preparation of PCR reaction reagents

Reagents required for PCR reaction include A) Tag DNA polymerase (heat-resistant DNA polymerase), B) dNTPs (deoxynucleotides solution), C) PCR buffer (if MgCl<sub>2</sub> containing Mg<sup>2+</sup> is not included, MgCl<sub>2</sub> is separately required), and D) primers. If the primers from the method published in the AOAC Journal (Table 1) are used, general composition of PCR reaction solution is summarized in Table 3.

Table 3. Composition of PCR reaction solution

Components	Stock Soln.	Final conc. (tube)	Volume (for 1 reaction)
DNA polymerase*	5U/.	0.625U	0.125.
Buffer	10 x	1x	2.5.
MgCl <sub>2</sub> **	25mM	1.5mM	1.5.
dNTPs	2.5mM	200μM	2.
Primer	25μM, each	0.5μM, each	0.5.
Template DNA	20ng/.	50ng	2.5.
Sterilized DW		added to make the final volume of 25.	15.875.
Total			25.

\* For DNA polymerase, use Taq DNA polymerase that is used after activation at 95 °C for 10 minutes.

\*\* MgCl<sub>2</sub> concentration greatly affects the specificity and output of amplification. In general, 1.5 to 2.5mM is widely used. If Mg<sup>2+</sup> is in excess, non-specific PCR products are increased. If Mg<sup>2+</sup> is insufficient, PCR products are decreased.

### ③ Preparation of controls

Negative controls include A) those not containing DNA (to determine the contamination of PCR reaction system) and B) those not containing primers (to determine the contamination

of reaction system, except primers). Positive controls include C) those containing non-GMO DNA (to determine if extraction of DNA is correctly done, DNA extracted from 0% standard sample taken from the same agricultural product (products prepared by IRMM and sold by Fluka or other equivalent products)) and D) those containing target recombinant DNA to be detected (to determine if the PCR reaction system is correct, DNA extracted from 2% standard sample (products prepared by IRMM and sold by Fluka or other equivalent products)). Subject such negative and positive controls to PCR reaction together with the sample.

### (3) PCR

PCR reaction of each DNA extract is conducted in two confirmation tests according to the following procedure. In the first confirmation test, primers for internal standard gene and transcription initiation gene (promoter 35S) and/or transcription termination gene (NOS terminator) are used for PCR reaction. From this test, DNA extract that is found to have both amplification products (internal standard gene and 35 S promoter and/or NOS terminator) in more than one extracted solution out of two extraction samples is used as template DNA for the second confirmation test with primers for structural gene.

#### ① Preparation of master mixture

Determine the number of tubes to be used under consideration of the number of samples for PCR reaction and calculate the amount of master mixture based on the resultant total volume and some extra volume. Prepare the PCR reaction solution according to Table 3, except template DNA. Handle the master mixture on ice and dispense 22.5 µl to each PCR tube. For negative controls that do not use primers, take 22 µl of master mixture not containing primers and add 0.5 µl of sterilized distilled water.

#### ② Addition of template DNA

Add 2.5µl of template DNA solution to 22.5µl of master mixture. Conduct addition of template DNA in the order of negative controls, DNA extract (20ng/µl), and positive controls.

#### ③ PCR amplification

Conduct PCR after dispensing all reaction solutions. Reaction conditions for PCR depend on primers. Table 4 shows the reaction conditions when primers prepared and sold by Nippon Gene are used. After starting the first denaturation by keeping the mixture at 95 °C for 10 minutes, perform denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds as one cycle. Repeat this cycle 40 times. Then, perform final elongation at 72 °C for 7 minutes. When all reactions are completed, keep the mixture at 4 °C. Conduct electrophoresis to confirm the amplification results or store such PCR products at freezing condition.

Table 4. PCR reaction conditions

	Temperature	Time	Cycle
First denaturation	95 °C	10 min.	1 cycle
Denaturation	95 °C	30 sec.	40 cycle
Annealing	60 °C	30 sec.	
Extension	72 °C	30 sec.	
Elongation	72 °C	7 min.	1 cycle
Storage	4 °C	-	-

. If necessary, the above conditions may be adjusted.

#### (4) Electrophoresis and judgment

The results from PCR amplification can be confirmed by electrophoresis using agarose gel or polyacrylamide gel. The agarose gel electrophoresis method is described here.

##### ① Preparation of agarose gel

In this step, use of specially sterilized materials is not necessary, but it is desirable to wear rubber gloves to prevent any possible risks, such as contamination.

First, assemble the gel maker. The concentration of gel is determined under consideration of the length of DNA to be electrophoresed and the type of agarose gel. In this case, since DNA extracted from sample and PCR-amplified products of about 100 to 200 bp are subjected to electrophoresis, use the agarose gel suitable for this purpose according to the manufacturer's instruction. Add TAE (Tris-acetate/EDTA) buffer (0.5 x) to the required quantity of agarose, and heat the mixture to dissolve the agarose. Further, since PCR-amplified products of below 200 bp are subjected to electrophoresis, it is desirable to use the agarose of high gel strength. For the pre-staining method, cool the mixture to approximately 55 °C when the agarose is sufficiently dissolved to result in homogeneous gel, add 1µl ethidium bromide (EtBr) solution (10mg/ml) per 100ml of the above solution, and mix it well. When the gel is cooled down to about 55 °C, pour it into a gel maker, insert the comb, and leave it for about 30 minutes to sufficiently solidify it. When the gel is solidified, add TAE buffer (0.5 x) sufficiently to allow the gel to be soaked in the buffer and remove the comb carefully. Install the agarose gel in the electrophoresis bath and fill it with TAE buffer (0.5 x) enough to soak the upper side of the gel in the buffer.

##### ② Electrophoresis

Add 1/6 x gel loading buffer to the PCR amplified reactant and mix them well. Inject the mixture into the well of the gel carefully. To both end wells, inject the appropriate marker DNA to determine the size of the PCR amplified reactant. The volume should not exceed 10µl. After injection, conduct electrophoresis at a constant voltage of 50 to 100 V until the BPB (promophenol blue) in the gel loading buffer advances to the 1/2 . 2/3 position of the gel. Then, stop the electrophoresis and identify the electrophoretic pattern using the gel image analyzer.

##### ③ Staining of gel

For the post-staining method, transfer the electrophoresed gel to a container containing 5µl of ethidium bromide (EtBr) per 100ml of TAE buffer (0.5 x) (10mg/ml), place the container on a shaker, and perform staining for 20 to 30 minutes while shaking the container gently. Then, place the container in distilled water for about 30 minutes for de-staining. When pre-staining is used, the procedures specified in this section are not necessary. The electrophoresis results can be immediately determined.

##### Note

Ethidium bromide (EtBr) is a luminescent reagent to be inserted into the DNA double helix structure. It is a potent mutagenic material. Therefore, careful attention should be paid. Be sure to wear rubber gloves and mask when handling this reagent. It is desirable to use the dedicated device for treatment of waste EtBr. Otherwise, treat EtBr with activated charcoal and then, discard it. If necessary, use a specialized company for disposal of highly concentrated EtBr.

##### ④ Gel image analysis

Place the stained electrophoretic gel on a piece of plastic wrap spread on the stage of a gel image analyzer or a trans-illuminator and radiate ultraviolet rays. Identify the electrophoretic pattern using a CCD camera and judge the presence of the target band by comparing it with

the marker DNA. Store the electrophoretic results as image data. When a Polaroid camera is used, take a photo and store it.

(5) Judgement of results

When the target PCR amplified band is found in the lane, judge the results as follows:

① At first, PCR reactant should not be detected in negative controls (A - negative control not containing DNA and B - negative control not containing primer). Then, for the positive control containing non-GMO DNA (C), the PCR reactant for internal standard gene should be found, but the PCR reactant for recombinant DNA should not be detected. In addition, for the positive control containing target recombinant DNA, both PCR reactants for internal standard gene and recombinant DNA should be confirmed. If one of the above criteria is not met, conduct re-tests.

② If PCR analysis of two DNA extracts (from repeated extraction processes) shows PCR reactants specific to internal standard gene and recombinant DNA in more than one DNA extract through the first and second confirmation tests, it is determined as "detected". If the PCR reactant specific to internal standard gene is found and the PCR reactant specific to recombinant DNA is not detected, it is determined as "not detected". If the PCR reactant specific to internal standard gene is not detected in both DNA extract samples, conduct re-test from the process of DNA extraction. If the PCR amplified reactant specific to internal standard gene is not found in the re-test, it is determined as "impossible". (Refer to Table 5.)

③ In the GMO plants, RRS, Bt11, Bt176, T25, and Mon810 use 35S as the transcription initiation gene (promoter) and RRS, Bt11, and GA21 use NOS as the transcription termination gene (terminator).

④ If an agricultural product is judged as "detected" according to the above qualitative analysis, it will be subjected to examination of documents, including the classified distribution certificate, and labeling. Further, the quantitative analysis will be conducted for the relevant product in question. However, agricultural products unsuitable for foods or exempted from safety assessment, such as StarLink corns (CBH351), will not be subjected to such examination of documents, such as the classified distribution certificate, and quantitative analysis.

## 6) Quantitative analysis

At present, the PCR-based quantitative analysis of foods produced by recombinant DNA techniques does not give the absolute quantity value. However, it is possible to measure the relative quantity of recombinant DNA present in such foods from the ratio of recombinant DNA to internal standard gene according to the method published in the AOAC Journal.

"Real-Time PCR" method requires DNA probes having the base sequence complementary to template DNA and binding luminescent material as well as primers for reaction of polymerase used in general PCR method. As polymerase reaction continues, these come to specifically bind with the increasing reactants and luminescent materials become separated from probes. The amount of such separated luminescent materials are dynamically measured and the results are used to calculate the amount of template DNA.

Further, the quantitative PCR method includes the competitive PCR not requiring any special devices and the method utilizing the standard curve obtained using reference standard samples.

Table 5. Flow chart of judgment (example)

Samples	DNA Extracts	1st confirmation		2nd confirmation	GMO Judgement
		Internal standard gene	P35S, tNOS	Structural gene	
Sample No. 1	Extraction 1	+	+	+	Detected
	Extraction 2	+	+	+	
Sample No. 2	Extraction 1	+	+	+	Detected
	Extraction 2	+	+	-	
Sample No. 3	Extraction 1	+	+	+	Detected
	Extraction 2	+	-	/	
Sample No. 4	Extraction 1	+	+	+	Detected
	Extraction 2	-	-	/	
Sample No. 5	Extraction 1	+	+	-	Not detected
	Extraction 2	+	+	-	
Sample No. 6	Extraction 1	+	+	-	Not detected
	Extraction 2	+	-	/	
Sample No. 7	Extraction 1	+	+	-	Not detected
	Extraction 2	-	-	/	
Sample No. 8	Extraction 1	+	-	/	Not detected
	Extraction 2	+	-	/	
Sample No. 9	Extraction 1	+	-	/	Not detected
	Extraction 2	-	-	/	
Sample No. 10	Extraction 1	-	/	/	Impossible
	Extraction 2	-	/	/	

+ : Specific band detected

- : Specific band not detected

/ : Test not required

1. Extraction is conducted two times for one sample to perform PCR reaction. For Sample No. 10, one extraction is additionally conducted.
2. Depending on the GM varieties, 35 S promoter or NOS terminator may not be detected.
3. If primers sold by Nippon Gene (Japan) are used for PCR test of potatoes or StarLink corns (CBH351), use the primers for the first detection instead of the promoter and the terminator.

#### (1) Real-time PCR

This quantitative PCR method using TaqMan Chemistry employs a luminescent oligonucleotide probe, which anneals in the DNA sequence between the both primer pairs (3' and 5') and has two dyes (reporter and quencher). When this probe is hydrolyzed by the 5'-nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher dye and emits luminescence. The strength of luminescence shows exponential increase for the PCR cycle. Therefore, by comparing the PCR cycle number which has reached a certain luminescence, the amount of original DNA can be obtained.

The quantitative analysis of GM products is to determine the amount of recombinant gene compared to the internal standard gene which is the universally present gene in the non-GMOs.

#### (2) Quantification of soybeans produced by recombinant DNA techniques

In the case of soybeans, use the lectin-gene that is ubiquitous in soybeans as the internal standard gene. The number of copies of the lectin-gene in the DNA sample is obtained by quantitative PCR which uses a primer pair (Le1-n02) detecting the lectin gene and a probe (Le1-Taq) detecting the recombinant gene. At the same time, the number of copies of the recombinant gene in the sample is obtained by another quantitative PCR using a primer pair

(RRS-01) and probe (RRS-Taq). The percentage of GMO in sample is obtained from the following way. Divide the number of recombinant gene copies by the number of the lectin gene copies. And divide this value again by the internal standard ratio (which is determined previously), and multiply the result by 100.

(3) Quantification of corns produced by recombinant DNA techniques

In the case of corns, there are many GM varieties (Bt176, Bt11, T25, Mon810, GA21) that have different target proteins. Therefore, quantify each GM variety separately, based on the qualitative analysis results and judge from the result of total of the quantitative value for each GM variety.

The analysis method is similar to that for quantitative PCR of soybeans, except the primer pair and probe to be used. In the case of corn, use the starch synthase IIb (SSIIb) gene as the universally present internal standard gene. Calculate the amount of GMO content using the number of copies of starch synthase IIb (SSIIb) gene (obtained from the primer pair SSIIb-3 and probe SSIIb-Taq) and the number of copies of target recombinant gene (obtained from the primer pair and probe, which detects target recombinant gene).

Bt176, Bt11, T25, MON810, and GA21 use Bt176-2 and Bt176-Taq, Bt11-3 and Bt11-Taq, T25-1 and T25-Taq, M810-2 and M810-Taq, and GA21-3 and GA21-Taq as primer pair and probe, respectively.

Internal standard ratios applicable to GM soybeans and corns depend on the type of Real-Time PCR equipment and they are summarized in Table 6, Table 7, Table 8, and Table 9.

Table 6. Quantitative internal standard ratios for GM soybeans and corns (ABI PRISM™ 7700 & 5700)

Products	Varieties	Internal standard ratios	Remarks
Corn	35S promoter screening	0.39	SSIIb-3 and SSIIb-Taq P35S-1 and P35S-Taq
	Bt11	0.44	SSIIb-3 and SSIIb-Taq Bt11-3 and Bt11-Taq
	GA21	2.01	SSIIb-3 and SSIIb-Taq GA21-3 and GA21-Taq
	T25	0.34	SSIIb-3 and SSIIb-Taq T25-1 and T25-Taq
	Bt176	1.99	SSIIb-3 and SSIIb-Taq Bt176-2 and Bt176-Taq
	MON810	0.38	SSIIb-3 and SSIIb-Taq Mon810-2 and Mon810-Taq
Soybean	40-3-2 (RRS)	1.05	Le1-n02 and Le1-Taq RRS-01 and RRS-Taq

Table 7. Quantitative internal standard ratios for GM soybeans and corns (ABI PRISM™ 7900HT 96well)

Products	Varieties	Internal standard ratios	Remarks
Corn	35S promoter screening	0.38	SSIIb-3 and SSIIb-Taq P35S-1 and P35S-Taq
	Bt11	0.40	SSIIb-3 and SSIIb-Taq Bt11-3 and Bt11-Taq
	GA21	1.99	SSIIb-3 and SSIIb-Taq GA21-3 and GA21-Taq
	T25	0.34	SSIIb-3 and SSIIb-Taq T25-1 and T25-Taq
	Bt176	2.02	SSIIb-3 and SSIIb-Taq Bt176-2 and Bt176-Taq
	MON810	0.36	SSIIb-3 and SSIIb-Taq Mon810-2 and Mon810-Taq
Soybean	40-3-2 (RRS)	1.04	Le1-n02 and Le1-Taq RRS-01 and RRS-Taq

Table 8. Quantitative internal standard ratios for GM soybeans and corns (ABI PRISM™ 7900HT 384well)

Products	Varieties	Internal standard ratios	Remarks
Corn	35S promoter screening	0.39	SSIIb-3 and SSIIb-Taq P35S-1 and P35S-Taq
	Bt11	0.43	SSIIb-3 and SSIIb-Taq Bt11-3 and Bt11-Taq
	GA21	2.06	SSIIb-3 and SSIIb-Taq GA21-3 and GA21-Taq
	T25	0.37	SSIIb-3 and SSIIb-Taq T25-1 and T25-Taq
	Bt176	2.12	SSIIb-3 and SSIIb-Taq Bt176-2 and Bt176-Taq
	MON810	0.38	SSIIb-3 and SSIIb-Taq Mon810-2 and Mon810-Taq
Soybean	40-3-2 (RRS)	1.00	Le1-n02 and Le1-Taq RRS-01 and RRS-Taq

Table 9. Quantitative internal standard ratios for GM soybeans and corns (LightCycler)

Products	Varieties	Internal standard ratios	Remarks
Corn	35S promoter screening	0.53	SSIIb-3 and SSIIb-Taq P35S-1 and P35S-Taq
	Bt11	0.63	SSIIb-3 and SSIIb-Taq Bt11-3 and Bt11-Taq
	GA21	2.63	SSIIb-3 and SSIIb-Taq GA21-3 and GA21-Taq
	T25	0.31	SSIIb-3 and SSIIb-Taq T25-1 and T25-Taq
	Bt176	2.60	SSIIb-3 and SSIIb-Taq Bt176-2 and Bt176-Taq
	MON810	0.49	SSIIb-3 and SSIIb-Taq Mon810-2 and Mon810-Taq
Soybean	40-3-2 (RRS)	1.01	Le1-n02 and Le1-Taq

			RRS-01 and RRS-Taq
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(4) Standard solution for the standard curve

Prepare GM plasmid DNA solution of which the number of copies has already been determined and use it as the standard solution. Dilute the GM plasmid DNA solution with *Escherichia coli* ColE1/TE DNA solution (5 ng/μl) so as to make appropriate copy numbers for the standard curve (e.g. 20, 125, 1,500, 20,000, and 250,000 copies per well).

(5) Preparation of the solution for the PCR reaction

Dissolve all reagents used in quantitative PCR at room temperature and sufficiently mix them using a mixer. If the same tip is used successively for dividing, the usual handling will not work from the second time onward because the air is cooled in the pipette. It is necessary to read up on how to use the pipette at low temperature in the manual of the pipette before starting the procedure. Further, when handling high viscous reagents, such as Universal PCR Master Mix, place the pipette vertical to the solution surface, slowly push the button until the feeling of stopping is firstly felt, push it deeper a lot, relieve the button very slowly to suck Taq polymerase, push the button again slowly to discharge the solution until the feeling of stopping is firstly felt, and finally relieve the button very slowly to suck the solution. On dispensing, place the pipette vertically, push the button slowly to dispense the solution until the feeling of stopping is firstly felt, remove the pipette from the tube, and then, discard the tip.

25μl solution for the PCR reaction contains 12.5μl of Universal PCR Master Mix, 0.5μl of primer (25mM each), 0.5μl of probe (10mM), 9μl of sterilized distilled water, 2.5μl of 20ng/μl DNA sample solution (50ng), and 2.5μl of the standard solution for the standard curve or 2.5μl of 5ng/μl ColE1/TE buffer. 5ng/μl ColE1/TE buffer corrects the effect of trace DNA being non-specifically absorbed onto the wall surface of the tube and is used as negative control.

① Preparation of primer-probe mixture

Dilute primer and probe with sterilized distilled water to make the final concentration of 1.25mM and 0.5mM, respectively, and mix them well using a mixer.

② Preparation of Master Mix

Mix the primer-probe solution and Universal PCR Master Mix at the ratio of 1:1.25 using a mixer. In order to minimize the differences between PCR reactions, prepare PCR reaction solution for 3 wells per each DNA sample solution. The volume of the Master Mix per each DNA sample should be 81μl.

③ Dispensing of Master Mix

Prepare 500μl tubes for template DNA (the number of samples), standard solutions for the standard curve (5 per primer), and negative control solutions (1 per primer), and number them. Dispense the above mix solution of 78.75 μl into each 500μl tube.

④ Addition of template DNA, standard solutions for the standard curve, or negative control solutions into the above tubes containing master mix.

To tubes containing master mix, add 8.75μl of template DNA (20ng/μl), 8.75μl of standard solutions for the standard curve, or 8.75μl of negative control solutions. Use a mixer to mix them well. Dispense 25μl of the mix solution to each well. Put the lid on the plate. At this time, to be impartial, close alternately from both sides. Then, using a special roller, seal up the wells. Lastly, observe the bottom of the wells, and if there are bubbles, knock lightly a rim of the plate or perform centrifugation (using the centrifuge for 96-well plate) to remove bubbles.

## (6) Quantitative PCR

Quantitative PCR is performed using the Real-Time PCR device. Set the plate onto the device, input well data, check if the cover temperature is around 105 °C, and begin the reaction. The reaction conditions are as follows: After maintaining it at 50 °C for 2 minutes, keep it at 95 °C for 10 minutes. After starting the reaction with a hot start method, conduct 40 cycles of amplification reaction (30 seconds at 95 °C and 1 minute at 59 °C for 1 cycle). After the end of the reaction, analyze the results.

## (7) Preparation of the standard curve

The standard curve for the internal standard gene and the recombinant gene can be obtained from the following way.

The following description is an example of preparing the standard curve when ABI PRISM 7700 is used. If other types of equipment are used, prepare the standard curve according to the relevant user's manual. On the amplification curve plotting the increasing amount of luminescent signals ( $\Delta Rn$ ) against the number of reactions, select the  $\Delta Rn$  part where luminescent signals from both of the standard solution for the standard curve and DNA sample solution amplify exponentially, and draw a threshold line (Th. line). At this time, the threshold line must not cross the non-specific amplified curve from the negative control solution (blank sample solution using CoE1 DNA solution). In addition, determine Th. line through the following manipulation on the window of "Amplification Plot". ① Set 3 for Start and 15 for Stop of Baseline. ② Enter the value of  $2^m$  to Mult\*Stddev. At first, m is 0 ( $m=0$ ). (In other words, enter the value of 1.) ③ Push "Suggest" button and then, "Update Calculations" button. ④ Select "Analysis" and "Standard Curve", check the correlation coefficient, slope, and Y-axis values of the standard curve, and enter them into "Th. line Decision Table" together with the value of Th. line. Increase the value of m by 1 and repeat this procedure. If one of NTC amplification curves crosses Th. line (when it is not possible to identify such cross by visual examination, it is determined as "crossed" if Ct value is 40 (in the case of 40-cycle PCR) in Experiment Report), write "NTC" in the remark. ⑤ Repeat this procedure until the standard amplification curve for the maximum number of copies used comes to cross Th. line. ⑥ If one of standard amplification curves for the maximum number of copies crosses Th. line, write "plot out" in the remark of "Th. line Decision Table". ⑦ Calculate the amplification rate (A) and  $\Delta A$ , and select Th. line satisfying the following conditions.

Definitions of A &  $\Delta A$ :  $A = 10^{(-1 / \text{slop})}$ ,  $\Delta A = (A_{m+1} \cdot A_m) / A_m \times 100$

Condition 1: Select Th. line at the midpoint in the section where  $\Delta A$  of more than 2 sections is below 1% (min. n, max. m). However, if the selected Th. line is not an integer, round the decimals. In this case, the correlation coefficient of the standard curve obtained from the selected Th. line should be above 0.99, the amplification rate (A) should be below 2.1, and the selected Th. line should not cross the NTC curve.

Condition 2: If the above Condition 1 is not met, adjust the acceptable value of  $\Delta A$  to 2%. If not met even after such adjustment, adjust the acceptable value of  $\Delta A$  to 3%, 4%, or 5%.

Condition 3: If there are several m values satisfying Condition 1 or Condition 2, select the largest m value.

Retest: If it is not able to determine Th. line even when 5% is employed for  $\Delta A$ , conduct re-test.

Use the point, where the Th. line and the luminescent signal of the standard solution for the standard curve cross, as the "threshold cycle (Ct)" and calculate the number of copies from

Experiment Report. Next, plot the Ct value for the logarithm of copies of the (each) standard solutions for standard curve (x-axis). Use the approximate straight line obtained from each Ct as the standard curve.

(8) Calculation of the content of the foods produced by recombinant DNA techniques in samples

Obtain the Ct value for unknown DNA sample, by using the Th. line of the standard curve. For each of the internal standard gene and recombinant gene, calculate the numbers of original genes' copies for all 3 wells from the standard curve. Use the average of these values as the number of copies of the original internal standard gene and original recombinant gene. Calculate the amount of GMO contained in the products from the following formula.

$$\text{GMO content in products (\%)} = \frac{\text{No. of copies (recombinant gene)}}{\text{No. of copies (internal standard gene)}} \times \frac{1}{\text{Internal standard ratio}} \times 100$$

In the case of soybeans, the relevant GM variety is only "Roundup Ready Soybean", the herbicide-resistant one developed by Monsanto, and the percentage of GMO in products can be calculated from the number of copies of Le-1 and RRS genes.

(9) Judgement of results

For each DNA sample extracted two times, calculate total content of RRS, GA21, Bt176, Bt11, T25, and MON810 from 6)-(2) and (3). If the average is more than 3%, it is concluded that classified distribution has possibly failed.

#### \* For information

You can purchase GM standard plasmid DNA used in preparation of the standard solution for the standard curve from Nippon Gene Co. (FASMARK Co.) or Wako Co. In addition, you can purchase the primer pairs and probes used in preparation of the solution for the PCR reaction from those companies.

### 7) Analysis of StarLink Corn (CHB351)

Import and distribution of StarLink corn is prohibited in accordance with Item No. 6 in Article 4 of the Food Sanitation Act. For raw corns and ground corns, firstly conduct the screening process with use of any commercially available kits (Trait Bt9 Lateral Flow Test kit, Cry9C QuickStix kit, or others) according to the lateral flow strip method. If the screening result is positive, conduct the PCR reaction for confirmation. For other processed foods, use the PCR method to determine the presence of GM corns.

(1) Lateral flow strip method

Perform the test according to the manufacturer's instruction.

(2) PCR method

① DNA extraction

Use DNA solution obtained from repeated extraction (2 times) according to the method described in 3) Extraction and purification of DNA and perform PCR reaction according to the qualitative PCR method described in 5) Qualitative analysis method. Use the following primers.

② Primer for detection of CBH351

A. Use SSI1b-1 primer to determine the internal standard gene.

B. Primer for 1st detection of CBH351

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'

C. Primer for 2nd detection of CBH351

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) Judgement of results

If the PCR amplified reactant of 151 bp is detected for SSIIb-1 primer to determine the internal standard gene and the PCR amplified reactant of 170 bp is detected for the primer for 1st detection of CBH351, conduct PCR reaction of the same DNA extract with use of the primer for 2nd detection of CBH351. If the PCR amplified reactant of 171 bp is confirmed from this PCR reaction, it is judged as "CBH351 detected". If one of two DNA extract solutions is judged as "CBH351 detected", the relevant sample is finally judged as "CBH351 detected". If one of two DNA extract solutions does not show the PCR amplified reactant corresponding to the internal standard gene, conduct electrophoresis again to confirm the PCR amplified reactant. If the PCR amplified reactant for the internal standard gene is not detected again, ignore the result obtained from the relevant extraction and use the result from another DNA extract for judgment. If the PCR amplified reactant for internal standard gene is not detected in both DNA extracts, conduct DNA extraction and PCR reaction again. If the PCR amplified reactant for the internal standard gene is not detected in the re-extracted DNA solution, it is judged as "Impossible". Table 10 shows the example of judgment.

**8) Analysis of GM potatoes (New Leaf Plus, New Leaf Y)**

For GM potatoes, use DNA solution obtained from repeated extraction (2 times) according to the method described in ? Potatoes and processed potato products, 3) Extraction and purification of DNA and perform PCR reaction according to the qualitative PCR method described in 5) Qualitative analysis method. Use the following primers. Primers for this test are prepared and sold by Nippon Gene (Refer to Table 2). Other primers that produce the equivalent results can be used.

(1) Primer for detection of New Leaf Plus

① Use Pss 01n primer to identify the internal standard gene.

F-primer (Pss 01n-5'): 5'-TGA CCT GGA CAC CAC AGT TAT-3'

R-primer (Pss 01n-3'): 5'-GTG GAT TTC AGG AGT TCT TCG A-3'

② Primer for 1st detection of New Leaf Plus

F-primer (p-FMV02-5'): 5'-AAA TAA CGT GGA AAA GAG CTG TCC TGA-3'

R-primer (PLRV01-3'): 5'-AAA AGA GCG GCA TAT GCG GTA AAT CTG-3'

③ Use PLRV-rep1-5' and PLRV-rep1-3' as primer for 2nd detection of New Leaf Plus.

(2) Primer for detection of New Leaf Y

① Use Pss 01n primer to identify the internal standard gene.

F-primer (Pss 01n-5'): 5'-TGA CCT GGA CAC CAC AGT TAT-3'

R-primer (Pss 01n-3'): 5'-GTG GAT TTC AGG AGT TCT TCG A-3'

② Use p-FMV05-5' and PVY02-3' as primer for 1st detection of New Leaf Y.

③ Use pVY01-5' and pVY01 -3' as primer for 2nd detection of New Leaf Y.

(3) Judgement of results

If the PCR amplified reactant of 216 bp is detected for the primer to determine the internal standard gene and the PCR amplified reactant of 234 bp and/or 225 bp is detected for the primer for 1st detection of New Leaf Plus and/or New Leaf Y, conduct PCR reaction of the same DNA extract with use of the primer for 2nd detection of New Leaf Plus and/or New Leaf Y. If the PCR amplified reactant of 172 bp and/or 161 bp is confirmed from this PCR reaction, it is judged as "New Leaf Plus and/or New Leaf Y detected". If one of two DNA extract solutions is judged as "New Leaf Plus and/or New Leaf Y detected", the relevant sample is finally judged as "New Leaf Plus and/or New Leaf Y detected". If one of two DNA

extract solutions does not show the PCR amplified reactant corresponding to the internal standard gene, conduct electrophoresis again to confirm the PCR amplified reactant. If the PCR amplified reactant for the internal standard gene is not detected again, ignore the result obtained from the relevant extract and use the result from another DNA extract for judgment. If the PCR amplified reactant for internal standard gene is not detected in both DNA extracts, conduct DNA extraction and PCR reaction again. If the PCR amplified reactant for the internal standard gene is not detected in the re-extracted DNA solution, it is judged as "Impossible". Table 10 shows the example of judgment.

Table 10. Example of judgment: StarLink corn (CBH351) and GM potato

Items		1	2	3	4	5	6	7	8	9
Extract 1	Internal Standard Gene	.	.	.	.	.	.	.	.	.
	1st detection	.	.	.	.	.	.	.	.	/
	2nd detection	.	.	.	.	/	/	.	.	/
Extract 2	Internal Standard Gene	.	.	.	.	.	.	.	.	.
	1st detection	.	.	.	.	.	.	.	.	/
	2nd detection	.	.	/	/	/	/	.	/	/
Judgement		Detected	Detected	Detected	Detected	Not Detected	Not Detected	Not Detected	Not Detected	/

\* Sample No. 9 will be subjected to a third extraction.

\*\* . means "detected", . means "not detected", and / means "test not required".