Korea Food and Drug Administration Notice #2004-174

Revised Standards and Specifications for Foods

(Proposal)

November 26, 2004

Korea Food and Drug Administration

KFDA Notice #2004-174

This Notice is to provide the public with information concerning the purpose and content of the proposed revision of the Standards and Specifications for Foods; and to solicit public opinions and comments thereupon pursuant to Article 46 of the Administrative Procedures Act.

November 26, 2004

Commissioner
Korea Food and Drug Administration

Revised standards and specifications for foods (proposal)

1. Purpose

The revision is intended to establish testing methods for genetically modified foods in order to ensure the effectiveness of the genetically modified food labeling regulations established in accordance with Article 10 Paragraph 1 of the Food Sanitation Act and the safety assessment regulations established in accordance with Article 15 Paragraph 1 of the Act.

2. Gist

A. The phrase "Labeling standards for genetically modified foods, etc." will be added to 1. 3) of Chapter 1 General
B. A new provision will be added to "Chapter 3. Common standards and specifications for foods in general 2. Requirements for raw materials, etc. 1) Food ingredients" to allow the use of raw materials which have passed the safety assessment review for genetically modified foods, etc.
C. "Testing methods for genetically modified foods" will be added under Chapter 7. General testing methods.

3. Submission of opinions

A person or organization who desires to make comments on this proposed revision is requested to submit a letter of opinions and comments containing the following to the KFDA Commissioner (Division of Nutrition Evaluation; address: #5 Nokbun-dong, Eunpyung-Gu, Seoul, Korea; telephone: 02-380-1677/80; fax: 02-380-1358) not later than December 2004. For more information, please visit the KFDA web site (www.kfda.go.kr).

1. Opinions on individual items of the proposed revision (pros and cons, and the reasons)
2. Name (for an organization, organization name and representative name), address, and telephone number.
3. Miscellaneous

Revised standards and specifications for foods (proposal)

The standards and specifications for foods shall be revised as follows:

Chapter 1. 1. 3) shall be revised as follows:

3) Labeling standards for foods/food additives and utensils/containers/packaging and genetically modified foods, etc. in accordance with Article 10 Paragraph 1 of the Food Sanitation Act

Chapter 3. 2. 1) (20) shall be added as follows:

(20) If agricultural/livestock/marine products, etc. grown/raised using genetic recombination technologies which take useful genes from the genome of an organism and combine them with the genome of another organism are to be used as raw materials, etc., such products shall be determined to be suitable as raw materials, etc. by a safety assessment/review conducted pursuant to the “regulations concerning genetically modified food safety assessment review, etc.” as provided under Article 15 Paragraph 1 of the Food Sanitation Act.

The following shall be added as Item 23 to Chapter 7. General testing methods:

23. Genetically modified food testing methods

GM food testing methods 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, etc. 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 and the quantitative analysis of the genes uses real-time PCR equipment. However, both qualitative and quantitative analytical methods are applicable to agricultural products and slightly processed agricultural products while only the qualitative analysis is applied to processed foods because quantitative analytical procedures are not yet established for processed foods.

As analytical techniques for GM foods continuously improve, analytical methods need continuous review and reassessment in consideration of advances in such techniques.

1) Sample preparation

(1) Washing and grinding of grains/pulses

Measure a grain or pulse sample of at lease 3,000 grains or 1 kg, wash it sufficiently in a 1% sodium dodecyl sulfate (SDS) solution by stirring it until froth is formed, remove the solution, and rinse it with distilled water ten times or more until the froth is removed. Let it dry naturally and grind it homogeneously with a grinder. Use the homogenously ground sample for analysis.

(2) Sample preparation from a processed food with high water content

In the case of a sample with high water content such as soybean milk, centrifuge it at 8,000xg for 15 minutes or leave it in a desiccator at 55? overnight. Grind the solid homogeneously. Use the ground sample for analysis.

(3) Sample preparation from a processed food with high sugar content

In the case of a sample with high sugar content such as confectionery products coated with syrup, etc., grind it with a grinder, melt the sugar of the sample with distilled water, and centrifuge it to obtain the solid. Repeat the procedure until sugar is removed sufficiently, and use the remaining solid for analysis.

(4) Sample preparation from a dry processed food

Highly absorbent processed foods may absorb DNA extraction reagents, interfering with DNA extraction. To prevent this, add an adequate amount of sterile distilled water to the sample, homogenize it with a grinder, centrifuge it at 8,000xg for 15 minutes at room temperature, and use the remaining solid for analysis.

2) Sample measurement and prevention of contamination

Clean the weighing balance and its vicinity with 70% ethanol before measurement to prevent contamination, lay a piece of plastic food wrap on the pan of the balance, and measure a sample in a tube to be used for DNA extraction. Use sterile tools during measurement. Use different tools for different samples, but, if inevitable, the same tools may be used after cleaned with 70% ethanol. Clean the mouth of the tube after measurement and plug the tube with a stopper.

3) DNA extraction/purification

The CTAB method which extracts/purifies DNA with a mixture of the surfactant cetyltrimethyl ammonium bromide (CTAB) and phenol chloroform is widely used in various applications and has the advantage of leaving little PCR inhibitory material, making it possible to obtain highly purified DNA. However, the method has disadvantages including the use of hazardous reagents such as phenol and chloroform and the complexity of operation.

To avoid such disadvantages, commercial DNA extraction kits may be used. Commercial DNA extraction kits include kits using silica gel membrane (QIAGEN Plant Maxi kit, QIAGEN DNeasy Plant Mini kit, etc.), kits using silica-based resins (Promega Wizard DNA Clean-Up System, etc.), and kits using magnetic particles (Promega Wizard Magnetic DNA Purification System for Food, etc.).

Using these kits, DNA may be extracted/purified from soy, corn, potato, and their products for PCR analysis. DNA extraction shall be performed twice on a sample, PCR shall be conducted on each of the extracted DNA, and the results shall be analyzed.

(1) CTAB method

Load 2g of ground sample in a polypropylene tube (50?), add 15? of CTAB buffer, mix the solution well using a vortex mixer, add 30? of the CTAB buffer additionally to the solution, and let the solution stand at 55? for 30 minutes. Put 600? of the solution sufficiently homogenized with a vortex mixer in a 1.5? tube, add 500? of a phenol-chloroform mixture to the solution, mix it well, and centrifuge the solution at 7,500xg at room temperature for 15 minutes. Transfer the upper aqueous phase to a new 1.5? tube, add 500? of chloroform-isoamyl alcohol mixture to the solution, mix the mixture homogeneously with a vortex mixer, and centrifuge the mixture at 7,500xg at room temperature for 15 minutes. Again transfer the upper aqueous phase to another 1.5? tube, add the same amount of chloroform-isoamyl alcohol mixture to the solution, turn the tube upside down about

ten times, and centrifuge the solution at 7,500xg at room temperature for 10 minutes. Discard the clear upper aqueous phase, add 500? of 70% ethanol to the precipitate slowly along the wall of the tube, centrifuge the tube at 7,500xg at room temperature for 1 minute, remove the upper aqueous phase with a micropipette without touching the precipitate, and dry the precipitate. Make sure that the precipitate be dried completely. Add 50? of TE buffer (pH 8.0) to the precipitate, mix the mixture well, and leave the precipitate to dissolve in the buffer at room temperature for 15 minutes, shaking it from time to time. (If the precipitate does not dissolve well, leave it at 4? for 12~24 hours until it is dissolved completely.) To purify extracted DNA, add 5? of RNase A (10? /?) to the solution, and leave it at 37? for 30 minutes. Add 200? of CTAB buffer and then add 250? of chloroform-isoamyl alcohol mixture to the solution, mix the mixture lightly with a vortex mixer, centrifuge the solution at 7,500xg at room temperature for 15 minutes, and transfer the upper aqueous phase into another 1.5? tube. When transferring the aqueous phase, make sure not to touch the middle layer of the solution. Add 200? of isopropyl alcohol to the liquid, turn the liquid upside down about 10 times, and centrifuge it at 7,500xg at room temperature for 10 minutes. Remove the upper aqueous phase with a micropipette without touching the precipitate, add 200? of 70% alcohol carefully along the wall of the tube to wash the precipitate, and again remove the upper aqueous phase carefully with a micropipette. Then, centrifuge the solution at 7,500xg at room temperature for 1 minute, remove the remaining ethanol with a micropipette, and dry the precipitate. Make sure not to dry the precipitate completely. Add 50? of sterile water or TE buffer (pH 8.0) to the dried precipitate, and leave the precipitate to dissolve completely at room temperature for 15 minutes, shaking it from time to time. Use the dissolved precipitate for DNA analysis. (If the precipitate does not dissolve well, leave it at 4? for 12~24 hours until it is dissolved completely.)

[Reagent preparation]

? CTAB buffer

Put 8? of 0.5M ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0), 20 ? of 1M tris-hydrochloric acid (HCl) buffer (pH 8.0), and 56? of 5M saline solution in a beaker, add distilled water until the solution becomes about 150? , add 4g of CTAB and dissolve it completely. Adjust the amount of the solution to 200? with distilled water, and sterilize the solution at 121? for 15 minutes.

? Phenol-chloroform mixture

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

? Chloroform-isoamyl alcohol mixture

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

? TE buffer (pH 8.0)

Prepare the buffer system by adding sterile distilled water to the final concentrations of 10mM and 1mM for tris-HCl solution (pH 8.0) and EDTA solution (pH 8.0) respectively.

(2) Extraction method using silica gel membranes

Extract DNA using the QIAGEN Plant Maxi kit or other kits of equivalent performance.

? Soy and soy products

If the QUIGEN Plant Maxi kit is used, load 1g of homogeneously ground sample in a polypropylene tube (50?), add 10? of AP1 buffer and 20? of RNase A (100? /?) which are preheated to 65? , mix the solution sufficiently with a mixer, and leave it to react at 65? for 1 hour. During the reaction, mix the mixture with a mixer for 10 seconds every 15 minutes so that the sample and the reaction mixture may be mixed sufficiently. After the 1-hour reaction, centrifuge the solution at 3,000xg at room temperature for 10 minutes, transfer the upper aqueous phase (7?) into another 50? tube without touching the membrane formed in the surface of the solution and the precipitate. Add 2.5? of AP2 buffer, leave the solution in ice for 15 minutes, and centrifuge the solution at 3,000xg at room temperature for 35 minutes. Load the clear upper aqueous phase (8?) onto the QIA shredder spin column, and centrifuge it at 3,000xg at room temperature for 5 minutes using a swing rotor. Transfer the liquid which has passed through the column (7.5?) to another 50? tube, mix it well for 10 seconds, and then transfer the liquid (6.8?) again to another 50? tube. Add AP3 solution containing ethanol 1.5x the liquid (10.2?), mix it sufficiently for 10 seconds, transfer the solution onto the DNeasy Maxi spin column, centrifuge the column at 3,000xg at room temperature for 5 minutes using a swing rotor to let the DNA bind to the column, and discard the remaining liquid which has passed through the column. Add 12? of AW solution to the column, and centrifuge the column at 3,000xg at room temperature for 15 minutes for washing, and transfer the column into another 50? tube, add 1? of sterile distilled water which is preheated to 65? , leave the tube at room temperature for 5 minutes, and elute DNA by centrifugation at 3,000xg at room temperature for 10 minutes using a swing rotor. Transfer 2? of eluted solution into a 2? tube, add the same amount of isopropyl alcohol to the solution, turn the tube upside down about 10 times, and leave it at room temperature for 5 minutes. Centrifuge the tube at 12,000xg at 4? for 15 minutes, and remove the upper aqueous phase with a micropipette without touching the precipitate. Add 500? of 70% ethanol to the tube and wash the precipitate carefully until the precipitate is separated from the wall of the tube. Centrifuge the tube at 12,000xg at 4? for 3 minutes, and remove the remaining ethanol with a micropipette, and dry the precipitate. Add 50? of sterile distilled water or TE buffer (pH 8.0) to the dried precipitate, and leave the precipitate at 4? for 12~24 hours until the precipitate is completely dissolved. Use the dissolved precipitate as the sample for DNA analysis.

? Corn and corn products

If the QUIGEN Plant Maxi kit is used, put 1g of homogeneously ground sample in a polypropylene tube (50?), add 5? of AP1 buffer and 10? of RNase A (100? /?) which are preheated to 65? , mix the solution sufficiently with a mixer, and leave it to react at 65? for 1 hour. During the reaction, mix the sample and the reaction mixture with a mixer for 10 seconds every 15 minutes so that the sample and the mixture may be mixed sufficiently. Add 1.8? of AP2 buffer, leave the solution in ice for 15 minutes, and centrifuge the solution at 3,000xg at room temperature for 15 minutes. Load the clear upper aqueous phase (4.2?) onto the QIA shredder spin column, and centrifuge it at 3,000xg at room temperature for 5 minutes using a swing rotor. Transfer the liquid which has passed through the column (4?) to another 50? tube, mix it well for 10 seconds, and then transfer the liquid (3.4?) again to another 50? tube. Add AP3 solution containing ethanol 1.5x the liquid (5.1?), mix it sufficiently for 10 seconds, load the solution onto the DNeasy Maxi spin column, centrifuge the column at 3,000xg at room temperature for 5 minutes using a swing rotor to let the DNA bind to the column, and discard the remaining liquid which has passed through the column. Add 12? of AW solution to the column, centrifuge the column at 3,000xg at room temperature for 15 minutes for washing, and transfer the column into another 50? tube, add 1? of sterile distilled water which is preheated to 65? , leave the tube at room temperature for 5 minutes, and elute DNA by centrifugation at 3,000xg at room temperature for 10 minutes using a swing rotor. Transfer 2? of eluted solution into a 2? tube, add the same amount of isopropyl alcohol to the solution, turn the tube upside down about 10 times, and leave it at room temperature for 5 minutes. Centrifuge the tube at 12,000xg at 4? for 15 minutes, and remove the upper aqueous phase with a micropipette without touching the precipitate. Add 500? of 70% ethanol to the tube and wash the precipitate carefully until the precipitate is separated from the wall of the tube. Centrifuge the tube at 12,000xg at 4? for 3 minutes, and remove the remaining ethanol with a micropipette, and dry the precipitate. Add 50? of sterile distilled water or TE buffer (pH 8.0) to the dried precipitate, and leave the precipitate at 4? for 12~24 hours until the precipitate is completely dissolved. Use the dissolved precipitate as the sample for DNA analysis.

? Potato and potato products

If the DNeasy Plant Mini kit is used, put 200? of homogeneously ground sample in a centrifugation tube (15?), add 1.5? of AP1 buffer and 10? of RNase A (100? /?) which are preheated to 65? , mix the mixture well with a mixer, and leave it to react at 65? for 15 minutes. During the reaction, mix the sample and the reaction mixture with a mixer for 10 seconds every 5 minutes so that the sample and the reaction mixture may be mixed sufficiently. Add 400 ? of AP2 buffer, leave the solution in ice for 5 minutes, and centrifuge the solution at 10,000xg at room temperature for 5 minutes. Transfer the clear upper aqueous phase into another centrifugation tube, load 500? of the liquid

onto the QIA shredder spin column, and centrifuge it at 10,000xg at room temperature for 2 minutes, and transfer the liquid which has passed through the column to another tube. Repeat the procedure with the remaining upper aqueous phase. Divide and transfer the liquid obtained from the procedure into two 2? tubes, add AP3 solution containing ethanol 1.5x the liquid, and mix it sufficiently for 10 seconds. Divide the liquid into 500? each, load it onto the same DNeasy Mini spin column, centrifuge the column at 10,000xg at room temperature for 1 minute to let the DNA bind to the column, and discard the lower aqueous phase which has passed through the column. Add 500? of AW solution to the column and centrifuge it at 10,000xg at room temperature for 1 minute. Add 500? of AW solution again, and repeat the same procedure. Centrifuge the column at 10,000xg at room temperature for 15 minutes to dry the column, and transfer the column into another 1.5? tube. Add 50? of sterile distilled water which is preheated to 65? , leave the tube at room temperature for 5 minutes, and elute DNA by centrifugation at 10,000xg at room temperature for 1 minute. Again add 50? of sterilized water which is preheated to 65? to the column, and repeat the previous procedure. Use the solution as the sample for DNA analysis.

? Note

AP1 and AP2 buffer and RNase A used in the QIAGEN Plant Maxi kit and the QIAGEN DNeasy Plant Mini kit, which are explained as kits using silica gel membrane technology under the section on DNA extraction/purification, are also available on the market besides those included in the kits.

4) Measurement of DNA concentration and purity, and condensation of the sample

To determine the DNA concentration, dilute the DNA sample with TE buffer (pH 8.0) adequately, measure the optical absorbance (optical density (O.D.)) at 260nm with a spectrophotometer, and calculate the DNA concentration (when the OD reading is 1, the DNA concentration will be 50ng/?). To determine the DNA purity, measure the OD each at 230, 260, and 280nm. If the OD_{260}/OD_{280} and OD_{260}/OD_{230} are in the range of 1.7~2.0, the DNA sample shall be judged to be suitable for a PCR analysis. If the OD_{260}/OD_{280} is below the range implying that the sample may be contaminated with impurities derived from proteins, the sample shall be treated with protease to recover DNA, and if the OD_{260}/OD_{230} is below the range, the sample shall be treated with amylase to recover DNA before a PCR analysis.

Dilute the DNA sample with TE buffer (pH 8.0) or sterilie distilled water to the concentration suitable for a PCR analysis. Shake the solution sufficiently to mix it and use the spun down solution as the sample for a PCR analysis. Put 20? of the solution each into 0.5 ? tubes, and store them below -20? . If samples are divided into smaller portions and kept frozen, gradually thaw them at room temperature, shake them to mix them, and use the spun-down solutions for analysis. If a sample is not consumed entirely in an analysis, do not use it for other analyses. If the DNA concentration is below the level required for PCR, perform DNA re-extraction. If the concentration is still below the PCR concentration even after re-extraction, use the

undiluted sample or perform the following condensation procedure.

To condensate the undiluted extracted DNA sample, add 3M sodium acetate (pH 5.2) 1/10x the sample (1/5x if10M ammonium acetate is used), add chilled ethanol 2 x the sample, and centrifuge it at 12,000xg for 15 minutes. (If isopropyl alcohol is used, add the same amount as that of the sample, and centrifuge it at ambient temperature.) Discard the upper aqueous phase after centrifugation, and wash the precipitate with 70% ethanol to remove salts and dissolve the precipitate in the adequate amount of TE buffer (pH 8.0) or sterile distilled water.

If the original DNA sample size is small, the sample size may be increased to 300~400? with sterile distilled water and then condensed.

5) Qualitative analytical methods

Qualitative analysis uses the standard PCR technique. It uses the DNA extracted from a sample as a template, amplifies DNA with primers of the targeted DNA, and identifies amplified DNA through electrophoresis.

Primers used in PCR to detect GM foods may be synthesized at the lab or obtained from commercial GMO detection kits. As even a small amount of a template DNA is amplified by PCR, testing instruments and reagents as well as the lab environment shall be carefully managed to prevent the sample from being contaminated by non-target DNA (particularly PCR amplification products). In addition, as DNA may be degraded by DNAses excreted from the surface of the human skin, all test tubes and tips shall be disposable products sterilized at 121? for 20 minutes or longer before use to prevent contamination with such enzymes. All solutions, etc. used for reagent preparation shall also be sterilized at 121? for 15 minutes or longer except for those which are not heat-stable. To minimize damage which may be caused by sterile distilled water or the TE buffer (pH 8.0) contaminated with DNAses, individual experimenters shall prepare water or the buffer separately in small portions as required by tests. DNA shall be handled in an isolated space such as in a clean bench. The top of a testing bench shall be cleaned with 70% ethanol and testing rubber gloves, etc. must be worn. Testing rubber gloves shall be those without powder on the surface or shall be used after powder on the surface is removed.

(1) Primer preparation

? Primers produced at the lab

Primers to detect GM soy, corn, and potato may be synthesized according to the AOAC methods provided in Table 1, and these primers are currently synthesized and marketed by Nippon Gene Co., Ltd. (Japan) (Table 2). Besides, Labs may design and synthesize primers of equivalent performance.

Table 1. Primers and probes used in AOAC methods

Purposes	Varieties	Primers/ probes	Base sequences
Endogenous genes	Soy	Le1n02-5'	5'-GCC CTC TAC TCC ACC CCC A-3'
		Le1n02-3'	5'-TCG ATT TCT CTC TTG GTG ACA GG-3'
		Le1-Taq	5'-FAM-AGC TTC GCC GCT TCC TTC AAC TTC AC-TAMRA-3'
	Corn	SSIIb 1-5'	5'-CTC CCA ATC CTT TGA CAT CTG C -3'
		SSIIb 1-3'	5'-TCG ATT TCT CTC TTG GTG ACA GG-3'
		SSIIb-Taq	5'-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3'
Screening	CMV p35S	P35S 1-5'	5'-ATT GAT GTG ATA TCT CCA CTG ACG T-3'
		P35S 1-3'	5'-CCT CTC CAA ATG AAA TGA ACT TCC T-3'
		P35S-Taq	5'-FAM-CCC ACT ATC CTT CGC AAG ACC CTT CCT-TAMRA-3'
	tNOS	NOS ter 2-5'	5'-GTC TTG CGA TGA TTA TCA TAT AAT TTC TG-3'
		NOS ter 2-3'	5'-CGC TAT ATT TTG TTT TCT ATC GCG T-3'
		NOS-Taq	5'-FAM-AGA TGG GTT TTT ATG ATT AGA GTC CCG CAA-TAMRA-3'
Structural genes	[Soy] RRS	RRS 01-5'	5'-CCT TTA GGA TTT CAG CAT CAG TGG-3'
		RRS 01-3'	5'-GAC TTG TCG CCG GGA ATG-3'
		RRS-Taq	5'-FAM-CGC AAC CGC CCG CAA ATC C-TAMRA-3'
	[Corn] Bt176	Bt176 2-5'	5'-TGT TCA CCA GCA GCA ACC AG-3'
		Bt176 2-3'	5'-ACT CCA CTT TGT GCA GAA CAG ATC T-3'
		Bt176-Taq	5'-FAM-CCG ACG TGA CCG ACT ACC ACA TCG A-TAMRA-3'
	Bt11	Bt11 3-5'	5'-AAA AGA CCA CAA CAA GCC GC-3'
		Bt11 3-3'	5'-CAA TGC GTT CTC CAC CAA GTA CT-3'
		Bt11-Taq	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 produced/marketed by Nippon Gene Co., Ltd. (Japan)

Target genes	Primer pairs	Amp. length	Remarks
[Soy]			
Endogenous genes	Le1n02-5'	118bp	*Le1*/sense
	Le1n02-3'		*Le1*/antisense
Recombinant genes	P35S1-5'	101bp	P35S/sense
(promoter)	P35S1-3'		P35S-pro/antisense
Recombinant genes	NOS ter 2-5'	151bp	tNOS/sense
(terminator)	NOS ter 2-3'		tNOS/antisense
Recombinant genes	RRS 01-5'	121bp	CTP4 from *P.hybrida*/sense
(structural gene)	RRS 01-3'		*EPSPS*/antisense
[Corn]			
Endogenous genes	SSIIb 1-5'	151bp	*zSSIIb*/sense
	SSIIb 1-3'		*zSSIIb*/antisense
Endogenous genes	SSIIb 3-5'	114bp	*zSSIIb*/sense
	SSIIb 3-3'		*zSSIIb*/antisense
Recombinant genes	P35S 1-5'	101bp	P35S/sense
(promoter)	P35S 1-3'		P35S/antisense
Recombinant genes	NOS ter 2-5'	151bp	tNOS/sense
(terminator)	NOS ter 2-3'		tNOS/antisense
Recombinant genes	Bt176 2-5'	100bp	*cryIA(b)*/sense
(For Bt176 detection)	Bt176 2-3'		PEPC#9 intron /antisense
Recombinant genes	Bt11 3-5'	127bp	*adh1*-1S/sense
(For Bt11 detection)	Bt11 3-3'		*cryIA(b)*/antisense
Recombinant genes	GA21 3-5'	133bp	OTP/sense
(For GA21 detection)	GA21 3-3'		*m-epsps*/antisense
Recombinant genes	T25 1-5'	149bp	*pat*/sense
(For T25 detection)	T25 1-3'		t35S/antisense
Recombinant genes	M810 2-5'	113bp	*hsp70* /sense
(For M810 detection)	M810 2-3'		*cryIA(b)*/antisense
Recombinant genes	CaM03-5'	170bp	CaM03/sense
(For initial CBH351 screening)	CBH02-3'		CBH02/antisense
Recombinant genes	Cry9C-5'	171bp	*cry9C*/sense
(For secondary CBH351 verification)	35Ster-3'		t35S/antisense
[Potato]			
Endogenous genes	Pss 01n-5'	216bp	*S.tuberosum* sucrose synthase/sense
	Pss 01n-3'		Same as above/antisense
Endogenous genes	UGPase01-5'	111bp	UGPase/sense
	UGPase01-5'		UGPase/antisense
Recombinant genes	p-FMV02-5'	234bp	p-FMV/sense
(For initial New Leaf Plus screening)	PLRV01-3'		PLRV/antisense
Recombinant genes	PLRV-rep1-5'	172bp	PLRV/sense
(For 2nd NewLeaf Plus verification)	PLRV-rep1-3'		PLRV/antisense
Recombinant genes	p-FMV05-5'	225bp	p-FMV/sense
(For initial NewLeaf Y screening)	PVY02-3'		PVY/antisense
Recombinant genes	pVY01-5'	161bp	PVY/sense
(For 2nd NewLeaf Y verification)	pVY01-3'		PVY/antisense

? Commercial GMO detection kits

Commercial kits may be used to detect GM soy, corn, and potato, provided that the results with such kits shall be equivalent to those obtained with the AOAC methods in Table 1. In addition, it is not suitable for an analysis of processed foods to design primers which generate 200bp or longer PCR products.

(2) PCR reagent preparation

Conduct PCR with necessary reagents including taq polymerase, etc. (Other polymerases with equivalent performance may be used.) Necessary reagents and procedures are listed below. To validate that genes are amplified with the extracted DNA serving as a template and targeted amplification products are obtained, a parallel PCR analysis shall be conducted with primers for endogenous genes corresponding to the target DNA sequences.

? Reference DNA solution preparation

Use DNA solutions extracted from 0% and 2% reference samples (products manufactured by IRMM and marketed by Fluka or equivalent products) as positive controls. In the case of PCR using the AOAC primers in Table 1, plasmids for positive control purposes produced and marketed by Nippon Gene Co., Ltd. (Japan) may be used instead of DNA extracted from a 2% reference sample.

? PCR reagent preparation

Reagents required for PCR reactions are (A) taq polymerase (heat-resistant DNA polymerase), (B) dNTPs (deoxynucleotide solution), (C) PCR buffer (If the buffer does not contain $MgCl_2$ containing Mg^{+2} ions, $MgCl_2$ is needed), and (D) primers, and when the AOAC primers in Table 1 are used, the general compositions of PCR reaction mixtures shall be as in Table 3.

Table 3. PCR reaction mixture set-up

Components	Stock solution concentration	Final concentration (tubes)	Unit volume
DNA polymerase*	5U/?	0.625U	0.125?
Buffer system	10x	1x	2.5?
$MgCl_2$**	25mM	1.5mM	1.5?
dNTPs	2.5mM	200µM	2?
Primers	25µM each	0.5µM each	0.5?
Template DNA	20ng/?	50ng	2.5?
Sterile distilled water		Added to make the total volume of 25?	15.875?
Total volume			25?

* Use AmpliTaq™ Gold DNA polymerase (a product from Applied Biosystems) or other

polymerases with equivalent performance.

** $MgCl_2$ concentration affects amplification specificity and yieldssignificantly. The concentration of 1.5~2.5mM is usually used. Generally if Mg^{2+} is excessive, non-specific PCR products increase while PCR yields decrease if Mg^{2+} is not sufficient.

? Preparation of controls

During a PCR analysis, perform parallel PCR analyses on negative controls such as (A) samples not containing DNA (to check the contamination of PCR reaction systems), and (B) samples not containing primers (to check the contamination of the reaction systems excluding primers), and on positive controls such as (C) samples containing non-GMO DNA (to check whether DNA extraction is performed correctly. DNA extracted from 0% reference samples of the same crop variety (products produced by IRMM and marketed by Fluka or equivalent products)), and (D) samples containing target recombinant DNA (to check whether PCR reaction is conducted accurately. DNA extracted from 2% reference samples (products produced by IRMM and marketed by Fluka or equivalent products) or plasmid for positive verification purposes produced and marketed by Nippon Gene Co., Ltd.).

(3) PCR

Conduct PCR analysis on extracted DNA in two stages in the following manner. During the initial identification test, conduct PCR with primers for endogenous genes and the promoter 35S and/or the NOS terminator. Using as the template the DNA section for which all target amplification products (endogenous genes and the 35S promoter and/or the terminator) are detected from one or more of the samples obtained from extraction repeated twice on the same sample, conduct the secondary verification test using primers for structural genes.

? Master mixture preparation (conduct the preparation on the ice)

Calculate the volume of the master mixture by first deciding on the number of tubes to be used in consideration of the number of PCR samples, and then adding allowance to the total volume required. Combine all the reaction mixture components except for the template DNA according to the PCR reaction mixture set-up in Table 3. Put 22.5? of the mixture respectively into PCR tubes. In the case of negative controls not containing primers, put 22? of master mixture with no primers into each PCR tube, and add 0.5? of sterile distilled water to the tubes.

? Addition of template DNA

Add 2.5? of template DNA solution to each of the tubes containing 22.5? of the master mixture. Add the template to the tubes in the order of negative controls, extracted DNA (20ng/?) and positive controls.

? PCR amplification

After dividing and placing all the reaction mixture, conduct the PCR analysis. PCR conditions may vary depending on the primers used. If primers from Nippon Gene are used, the reaction conditions are provided in Table 4. Let the mixture stand at 95? for 10 minutes for initial denaturation, and perform 40 cycles of denaturation at 95? for 30 seconds, annealing at 60? for 30 seconds, and extension at 72? for 30 seconds. Let the final elongation occur at 72? for 7 minutes at the end, and set the temperature to 4? when all reactions are completed. Conduct electrophoresis on the PCR products obtained from the procedure for assessment or freeze them for later use.

Table 4. PCR reaction conditions

	Temperature	Duration	Number of cycles
Initial denaturation	95?	10 minutes	1
Denaturation Annealing Extension	95? 60? 72?	30 seconds 30 seconds 30 seconds	40
Elongation	72?	7 minutes	1
Storage	4?	--	--

* The conditions may be changed if they are not optimal for specific PCR analyses.

(4) Analysis of electrophoretic results

PCR amplification results can be confirmed with electrophoresis using agarose gels or polyacrylamide gels. The method using agarose gels is explained below for illustration.

? Gel tank preparation

Wear test gloves to avoid contamination, etc. though special sterilized instruments are not needed at this stage.

Assemble the gel maker. Gel concentration depends on the length of DNA to be analyzed and the type of agarose gels. If the PCR product is 100~200bp in length as in this test, use agarose gel LO3™ (from Takara) or equivalent products and adjust the concentration to 1.8%. For confirmation of DNA extracted from a sample, adjust the concentration to 0.8%. Measure the amount of agarose required, add TAE (Tris-acetate/EDTA) buffer (0.5x) to the agarose, and melt the agarose by applying heat. As PCR products are relatively short with the size of 200bp or shorter, use high-strength agarose gels. In the case of a pre-staining method, let agarose melt sufficiently and homogeneously, cool it down to about 55? , add 1? of ethidium bromide solution (10? /?) per 100? , and mix it well. When the gel is cooled to about 55? , pour the gel into the gel maker, insert the comb, and let it stand for about 30 minutes for solidification. When the gel is solidified, submerge the

gel in TAE buffer (0.5x), and remove the comb carefully. Place the gel in the electrophoresis tank, and pour TAE buffer (0.5x) until the surface of the gel is sufficiently submerged.

? Electrophoresis

Mix the PCR products with a dye (gel loading buffer) 1/6x, load the samples into the wells of the gel carefully, and pour marker DNA in both end wells of the gel to help determine the sizes of the PCR products easily. In this case, the volume of each sample shall not exceed 10? . After loading the samples, conduct electrophoresis at 50~100V. When the bromophenol blue (BPB) contained in the dye has run 1/2~2/3 the length of the gel, stop electrophoresis, and analyze the results using an image system, etc.

? Gel staining

In the case of a post-staining method, put the gel immediately after electrophoresis in the dye made by mixing EtBr solution (10? /?) with TAE buffer (0.5X) in the ratio of 5? :100? , gently shake the container on the shaker for 20~30 minutes for staining, and bleach the gel in distilled water for about 30 minutes. In the case of a pre-staining method, this procedure is not necessary, and the results may be analyzed immediately.

[Caution]

Caution shall be exercised when handling an ethidium bromide-based reagent because the fluorescent reagent which inserts into the double helix of DNA is a powerful mutagen. During handling, rubber gloves and a mask must be worn. The waste solution shall be treated with an EtBr treatment instrument, but if it is not possible, the solution must be neutralized with sodium hypochlorite before disposal. If it is a solution of high concentration, hire a specialized treatment company for treatment and disposal.

? Result analysis

Place a piece of plastic food wrap on a spectroscopy instrument or transilluminator, place the stained gel on top of it, and irradiate the gel with ultraviolet rays. Confirm the electrophoretic patterns with a CCD camera, and check whether bands are formed at expected locations compared against marker DNA. Keep the results in the form of image data. If a Polaroid camera is used, take pictures of the gel.

(5) Decisions based on analytical results and their treatment

After checking through electrophoresis whether targeted PCR products are obtained, make the decisions based on the analytical results as follows:

? After confirming that PCR products are not detected from the negative controls (A) the sample containing no DNA and (B) the sample containing no primers, check whether the endogenous DNA PCR products are detected but GMO PCR products are not detected from the positive control

(C) the sample containing no-GMO DNA and whether both endogenous DNA and GMO DNA PCR products are detected from the positive control (D) the sample containing the target GMO DNA. When any of the analytical data does not conform to the results stated above, tests shall be conducted again.

? If both endogenous and GMO DNA specific PCR products are identified in the initial and secondary verification tests from one or both DNA samples obtained from extraction repeated twice on the same sample, the decision "Detected" shall be given. If endogenous DNA PCR products are identified but GMO DNA-specific PCR products are not identified from either of the DNA samples obtained from extraction repeated twice, the decision "Not-detected" shall be given. And If endogenous DNA PCR products are not identified, the test procedure shall be repeated from the DNA extraction step. If endogenous DNA PCR products are not identified again even after the repeated test, the decision "Not-testable" shall be given (Table 5).

? Among GMO plants, RRS, Bt11, Bt176, T25, and Mon810 use P35S as the promoter, and RRS, Bt11, and GA21 use tNOS as the terminator.

? If the decision "Detected" is given to a sample according to the qualitative analysis explained above, check whether the certificate of separated distribution is attached or not to determine whether labeling requirements are violated, and conduct a quantitative analysis if the sample is from an agricultural product. In the case of a product determined to be not suitable for eating such as StarLink corn (CBH351) or a product which has not gone through safety assessment review, verification of the certificate of separated distribution and a quantitative analysis shall not be conducted.

6) Quantitative analytical methods

Even with the latest PCR-based quantitative analytical technology, it is still not possible to determine the absolute quantity of GMO in a food, but it is possible to quantify the content of GMO indirectly according to the AOAC method from the ratio of GMO DNA to the endogenous DNA, which is always present in an agricultural product.

This method requires polymerase reaction primers as used in PCR, probes containing fluorescent dyes and complementary to the template DNA sequence, and a real-time PCR instrument which calculates the amount of template DNA by kinetically measuring the amount of fluorescent material separated from probes when the probes bind specifically to PCR products which increase as polymerase reactions increase.

Other PCR-based quantification methods include the competitive PCR method which does not require a specialized instrument and a method which refers to the quantification standard curve obtained from external standard samples.

Table 5. Decision-making matrix (illustration)

Cases	Extracted DNA	1st identification		2nd verification	Decision on GMO
		Endogenous DNA	P35S, tNOS	Structural DNA	
Case 1	Extract 1	+	+	+	Detected
	Extract 2	+	+	+	
Case 2	Extract 1	+	+	+	Detected
	Extract 2	+	+	-	
Case 3	Extract 1	+	+	+	Detected
	Extract 2	+	-	/	
Case 4	Extract 1	+	+	+	Detected
	Extract 2	-	-	/	
Case 5	Extract 1	+	+	-	Not-detected
	Extract 2	+	+	-	
Case 6	Extract 1	+	+	-	Not-detected
	Extract 2	+	-	/	
Case 7	Extract 1	+	+	-	Not-detected
	Extract 2	-	-	/	
Case 8	Extract 1	+	-	/	Not-detected
	Extract 2	+	-	/	
Case 9	Extract 1	+	-	/	Not-detected
	Extract 2	-	-	/	
Case 10	Extract 1	-	/	/	Not-testable
	Extract 2	-	/	/	

+: specific bands are detected.
-: specific bands are not detected.
/: Tests are not necessary.

1. Extraction shall be repeated twice on a sample for PCR analysis, but in the case of Case 10, extraction shall be conducted a third time for PCR analysis.
2. The promoter 35S and the terminator NOS may not be detected depending on GMO varieties.
3. If PCR is conducted with primers from Nippon Gene Co., Ltd. For potato and StarLink corn (CBH351), the primers used for initial detection shall be used instead of the promoters and terminators.

(1) Real-time PCR

The TaqMan chemistry-based quantification method utilizes a fluorescent oligo-nucleotide probe which combines the "reporter" and "quencher" dyes and binds to the DNA sequence of the 3' and 5' primer pair. When the probe is hydrolyzed when 5'-nuclease of the DNA polymerase is activated, the reporter is separated from the quencher and emits fluorescence. The strength of fluorescence increases exponentially relative to the number of PCR reactions. Therefore, the original DNA amount may be calculated by comparing the number of PCR reactions relative to the strength of fluorescence.

Quantitative GMO analysis uses the genes universally present in non-GMO as the internal standard genes, and quantification is conducted by calculating the amount of GMO genes relative to the amount of the internal standard genes.

(2) Quantification of GM soy

The lectin genes, which are universally present in soy, are used as the internal standard genes. Using the primer pair (Le1-n02) and the probe (Le1-Taq), which identify the lectin genes and GMO genes respectively, run quantitative PCR and calculate the number of copies from the sample. Divide the number of GMO copies by the number of lectin gene copies, again divide the quotient by the pre-defined coefficient (correction coefficient: the ratio of the number of GMO genes to the number of internal standard genes (lectin genes for soy) detected from quantitative PCR on seeds. When primer pairs and probes are used, the coefficients is defined for different soy varieties), and multiply the quotient by 100 to determine the percentage of GMO soy in a sample.

(3) Quantification of GM corn

There are various GM corn varieties (Bt176, Bt11, T25, Mon810, GA21), and a sample may contain muptiple GM varieties, making it necessary to identify multiple targets from a single sample. First, based on qualitative analysis results, quantify individual varieties, and total the quantities of individual GM corn varieties to make a decision.

The analytical method for GM corn is identical to the method explained above in the section on quantitative PCR method for soy except for primers and probes used. Use starch synthase IIb (SSIIb) genes as the internal standard genes, which are universally present in corn. Calculate the number of internal standard gene copies obtained by using the primer pair (SSIIB-3) and the probe (SSIIb-Taq) specific to these genes and the number of GMO gene copies obtained by using the primer pair and the probe specific to the GMO genes to quantify individual GMO corn varieties.

For the GMO varieties Bt176, Bt11, T25, Mon810, and GA21, respectively use the quantitative primer pairs and probes Bt176-2 and Bt176-Taq; Bt11-3 and Bt11-Tag; T25-1 and T25-Taq; M810-2 and M810-Taq; and GA21-3 and GA21-Taq.
The correction coefficients for GMO soy and corn varieties vary depending on

the real-time PCR instrument used, and the coefficients are provided in Tables 6, 7, 8, and 9.

Table 6. Quantitative correction coefficients for GM soy and corn (ABI PRISM™ 7700 & 5700)

Crops	Varieties	Correction coefficient	Remarks
Corn	P35S promoter screening	0.39	SSIIb-3 & SSIIb-Taq and P35S-1 & P35S-Taq used
	Bt11	0.44	SSIIb-3 & SSIIb-Taq and Bt11-3 & Bt11-Taq used
	GA21	2.01	SSIIb-3 & SSIIb-Taq and GA21-3 & GA21-Taq used
	T25	0.34	SSIIb-3 & SSIIb-Taq and T25-1 & T25-Taq used
	Bt176	1.99	SSIIb-3 & SSIIb-Taq and Bt176-2 & Bt176-Taq used
	Mon810	0.38	SSIIb-3 & SSIIb-Taq and Mon810-2 & Mon810-Taq used
Soy	40-3-2 (RRS)	1.05	Le1-n02 & Le1-Taq and RRS-01 & RRS-Taq used

Table 7. Quantitative correction coefficients for GM soy and corn (ABI PRISM™ 7900HT 96 wells)

Crops	Varieties	Correction coefficient	Remarks
Corn	P35S promoter screening	0.38	SSIIb-3 & SSIIb-Taq and P35S-1 & P35S-Taq used
	Bt11	0.40	SSIIb-3 & SSIIb-Taq and Bt11-3 & Bt11-Taq used
	GA21	1.99	SSIIb-3 & SSIIb-Taq and GA21-3 & GA21-Taq used
	T25	0.34	SSIIb-3 & SSIIb-Taq and T25-1 & T25-Taq used
	Bt176	2.02	SSIIb-3 & SSIIb-Taq and Bt176-2 & Bt176-Taq used
	Mon810	0.36	SSIIb-3 & SSIIb-Taq and Mon810-2 & Mon810-Taq used
Soy	40-3-2 (RRS)	1.04	Le1-n02 & Le1-Taq and RRS-01 & RRS-Taq used

Table 8. Quantitative correction coefficients for GM soy and corn (ABI PRISM™ 7900HT 384 wells)

Crops	Varieties	Correction coefficient	Remarks
Corn	P35S promoter screening	0.39	SSIIb-3 & SSIIb-Taq and P35S-1 & P35S-Taq used
	Bt11	0.43	SSIIb-3 & SSIIb-Taq and Bt11-3 & Bt11-Taq used
	GA21	2.06	SSIIb-3 & SSIIb-Taq and GA21-3 & GA21-Taq used
	T25	0.37	SSIIb-3 & SSIIb-Taq and T25-1 & T25-Taq used
	Bt176	2.12	SSIIb-3 & SSIIb-Taq and Bt176-2 & Bt176-Taq used
	Mon810	0.38	SSIIb-3 & SSIIb-Taq and Mon810-2 & Mon810-Taq used
Soy	40-3-2 (RRS)	1.00	Le1-n02 & Le1-Taq and RRS-01 & RRS-Taq used

Table 9. Quantitative correction coefficients for GM soy and corn (LightCycler)

Crops	Varieties	Correction coefficient	Remarks
Corn	P35S promoter screening	0.53	SSIIb-3 & SSIIb-Taq and P35S-1 & P35S-Taq used
	Bt11	0.63	SSIIb-3 & SSIIb-Taq and Bt11-3 & Bt11-Taq used
	GA21	2.63	SSIIb-3 & SSIIb-Taq and GA21-3 & GA21-Taq used
	T25	0.31	SSIIb-3 & SSIIb-Taq and T25-1 & T25-Taq used
	Bt176	2.60	SSIIb-3 & SSIIb-Taq and Bt176-2 & Bt176-Taq used
	Mon810	0.49	SSIIb-3 & SSIIb-Taq and Mon810-2 & Mon810-Taq used
Soy	40-3-2 (RRS)	1.01	Le1-n02 & Le1-Taq and RRS-01 & RRS-Taq used

(4) Standard solutions to develop a calibration curve

Use a plasmid DNA standard solution for each GM variety for which the number of copies is known as the standard solution to develop a calibration curve. Dilute the solution with Co1E1/TE DNA solution (5ng/?) of *Escherichia coli* so that the appropriate number of copies (e.g., 20, 125, 1,500, 20,000, and 250,000) may be obtained to develop the curve (marketed by Nippon Gene Co., Ltd.).

(5) Quantitative PCR reaction mixture preparation

All quantitative PCR reagents shall be thawed at room temperature, sufficiently mixed with a mixer, and spun down before use. If the same tip is used repeatedly to load a reagent, caution shall be exercised because the air in the pipette is chilled, making it difficult to handle the pipette accurately from the second time on. Familiarize with the pipette handling methods explained in the product manuals when handling low-temperature test materials. In addition, when handling a highly viscous reagent such as the Universal PCR Master Mix (from Applied Biosystems), set a pipette at the right angle to the surface of the liquid, press the button slowly until the first halt is felt, then press the button a little further, and release force from the button very slowly to draw in taq polymerase. Then release the liquid by pressing the button slowly until the first halt is felt. When loading the reagent, set the pipette vertically, press the button slowly until the first halt is felt, slowly remove the pipette from the tube, and discard the tip.

The unit volume of PCR reaction mixture is 25? , and consists of 12.5 ? of the Universal PCR Master Mix, 0.5? of the target primers (25mM each), 0.5? of the target probe (10mM), 9? of sterile distilled water and 2.5 ? of 20ng/? DNA solution (50ng), and 2.5 ? each of standard solution for calibration curve development or 2.5? of 5ng/? Co1E1/TE solution. 5ng/? Co1E1/TE solution is used to compensate the non-specific attachment of a small amount of DNA to the tube wall, and as the test solution for negative controls.

? Primer-probe mixture preparation

Mix the primers and the probe to a concentration of 1.25mM each for the primers and 0.5mM for the probe, dilute the mixture with sterile distilled water, and mix it well with a mixer.

? Master mix preparation

Mix the primer-probe mixture and the Universal PCR Master Mix 1:1.25 with a mixer. To reduce errors during PCR, prepare the PCR reaction mixture sufficient for 3 wells per DNA sample. The prepared amount shall be 81 ? per DNA sample, taking loss into account.

? Loading of master mix

Prepare 500? tubes in a sufficient number for template DNA (number of

samples), standard solutions for calibration curve development (5 per primer), test solutions for negative controls (1 per primer), and labels the tubes with numbers. Load 78.75? each of the above-said mixture into the 500? tubes.

? Addition of template DNA, calibration curve standard solutions or negative control solutions to the master mix

Add 8.75? each of template DNA (20ng/?), and standard solution for calibration curve development or test solution for negative controls to the master mix loaded into a tube. Mix it well with a mixer, and spin it down. Load 25? of the mixture into each well. When covering the plate, fit the cover diagonally in either direction to prevent tilting, and seal the wells with the cover using a specialized roller. Observe the wells from below, and if there are air bubbles in the bottom, tap the edge of the plate lightly or centrifuge it (with a centrifuge used for 96-well plates) to remove the air bubbles completely.

(6) Quantitative PCR

Conduct quantitative PCR utilizing ABI PRISM 7700, 7900, 5700, LightCycler or other systems based on the equivalent principles. Load the plate into the system, enter well information, start the reaction after confirming that the temperature of the system cover is about 105? . The reaction conditions are as follows: Keep the plate at 50? for 2 minutes, leave it stand at 95? for 10 minutes, and hot-start the reaction. Conduct 40 cycles of 30 seconds at 95? and 1 minute at 59? . After the reactions, interpret the analytical results.

(7) Calibration curve development

Develop the calibration curve for internal standard genes and GMO genes in the following manner. The following illustrates how to develop a calibration curve based on the results obtained from analysis conducted with ABI PRISM 7700. On the amplification curve which plots the increases in fluorescent signals relative to the number of reactions (? Rn), select the ? Rn section where fluorescent signals derived from the standard solutions for calibration curve development and DNA samples increase exponentially, and draw the threshold line (Th. line). Note that the line shall not cross the non-specific amplification curve which may be plotted for negative controls (blank samples using Co1E1 DNA solution). In addition, Set th. on the "Amplification Plot" window in the following procedure: ? Set the "Start" value of the "Baseline" to 3 and "Stop" to 15. ? Enter 2^m as the Mult*Stddev value. In the beginning, m should be "0" (m=0: that is, enter the value "1"). ? Press the "Suggest" button, and then press the "Update Calculations" button. ? Select "Analysis,""Standard Curve," check Corr., slope, and Y-intercept values on the standard curve, and enter the values with the th.line value in the "th.line determination table." Increasing m by the increment of 1, repeat this procedure. If any of NTC amplification curves crosses the th.line (IF it is difficult to discern the cross visually, refer to the Ct value in the experiment report. If the value is 40 (in the case of 40-cycle PCR), then the curves shall be considered to be crossed.), "NTC" shall be written down in the "Remarks" section. ? Repeat

the procedure until any of the standard amplification curves for the largest number of copies used in the test crosses the th. line. ? If any of the standard amplification curves crosses th. line, "plot out" shall be written down in the "Remarks" section of the "th.line determination table. ? Calculate the amplification rate (A) and l? AI, and select the th. line which meets the following conditions.

Definition of A and ? A: $A=10^{(-1/slop)}$, ? $A=(A_{m+1}-A_m)/A_m \times 100$

Condition 1:

Select the th.line at the mid point (mt) in the section where l? AI is 1% or lower in two consecutive sections (Minimum n Maximum m). If the th.line is not an integer, round up the decimals to the whole number.

If the Corr. value on the standard curve obtained from the th. line shall be 0.99 or higher, and the amplification rate (A) shall be 2.1 or lower, and the selected th.line shall not cross the NTC curve.

Condition 2:

If Condition 1 is not met, the allowance for l? AI shall be changed to 2%. If the condition is not met either, it shall be changed to 3%, 4%, and 5%.

Condition 3:

If multiple "m" values meet Condition 1 or Condition 2, use the largest m value.

(Rejection)

If the th.line cannot be determined even when the allowance for ł? AI is changed to 5%, carry out the test again.

Calculate the copy numbers from the experiment report using the point where the th. line and fluorescent signals of standard solutions for calibration curve development are crossed as the threshold cycle (Ct) value. Then plot the log values of the copy numbers of standard solutions for calibration curve development, and use the closest line obtained for individual Cts as the calibration curve.

(8) Calculation of the percentage of GMO varieties in a sample

With the th. used for calibration curve development, calculate the Ct value for an unknown DNA sample, and intrapolate the value in the calibration curves of internal standard genes and GMO genes. Using the mean value of the numbers of initial DNA copies in 3 wells as the numbers of initial internal standard DNA copies and initial GMO DNA copies, calculate the percentage of GMO varieties in a sample according to the following formula:

? GMO variety percentage (%) = number of GMO DNA copies/number of internal standard DNA copies x 1/correction coefficient x 100

As the herbicide-resistant Roundup Ready soy developed by Monsanto accounts for most of GM soy used, the percentage of GM soy may be obtained from the numbers of Le-1 and RRS gene copies.

(9) Result analysis and decision

If the mean value of quantities of RRS, GA21, Bt176, Bt11, T25, and Mon810 obtained according to 6) (2) and (3) from the DNA sample extracted twice exceeds 3%, a decision shall be made that the product is likely to be non-compliant with the requirement of separate distribution.

? Note

Standard GMO plasmid DNA used for calibration curve development may be procured from Nippon Gene Co. (Fasmark Co.) or Wako Co. as well as primers and probes used in PCR reaction mixture.

7) Analytical methods for StarLink corn (CHB351)

StarLink corn is corn whose importation, distribution, etc. are prohibited according to Article 4 Paragraph 6 of the Food Sanitation Act. As for raw corn and slightly processed corn products, conduct initial screening with the Trait Bt9 Lateral Flow Test kit (a kit from SDI) the a Cry9C QuickStix kit (from EnviroLogix), which are based on lateral flow strip techniques. If the results are positive, verification tests shall be conducted by PCR to determine whether the product contains StarLink. As for processed foods, a decision shall be made based on PCR results.

(1) Analysis using lateral flow strips

Conduct tests according to the operating manual of the purchased kit.

(2) PCR

? DNA extraction

Use DNA solutions obtained from extraction repeated twice on the same sample according to the DNA extraction and purification method in 3), and conduct quantitative PCR explained under 5) qualitative analytical methods with the following primers.

? Primers for CBH351 screening

? Use SSIIb-1 primer to identify endogenous genes.

? Primers for initial CBH351 detection are as follows:
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'

? Primers for secondary CBH351 verification 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) Result analysis and decision

If PCR products of 151bp are detected in the solution containing the SSIIb-1 endogenous DNA primer and PCR products of 170bp are detected in the solution containing the CBH351 screening primer, conduct PCR again on the same sample with the CBH351 verification primer. If PCR products of 171bp are confirmed, the decision "CBH351 detected" shall be given to the sample. If the decision "CBH351 detected" is given to any of the two DNA samples, the final decision "CBH351 detected" shall be given to the product in question. If PCR products derived from endogenous genes are not detected in one of the two DNA samples, conduct electrophoresis again to identify PCR products. If PCR products for endogenous genes are not detected even in the second analysis, the results from the solution shall be discarded and the decision shall be given based on the results from the other DNA solution. If PCR products for endogenous genes are detected in neither of the two DNA solutions, conduct DNA extraction and PCR again and make a decision. If endogenous gene PCR products are not detected in a third DNA solution, the decision "Not-testable" shall be given. An illustrative decision-making matrix is provided in Table 10.

8) Analytical methods for GM potato (New Leaf Plus, New Leaf Y)

For analysis of GM potato, use DNA solutions obtained from extraction conducted twice on a single sample according to 3) DNA extraction and purification ? analytical methods for potato and its processed products, and conduct PCR with the following primers according to qualitative PCR methods explained in 5) qualitative analytical methods. Currently GM potato primers are synthesized and marketed by Nippon Gene Co. (See Table 2), and labs may design and use their own primers of equivalent performance.

(1) Primers used to detect New Leaf Plus

? Use the Pss 01n as the endogenous DNA primers.

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'

? Primers for New Leaf Plus screening are as follows:

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'

? As New Leaf Plus verification primers, use PLRV-rep1-5' and PLRV-rep1-3'.

(2) Primers used to detect New Leaf Y

? Use the Pss 01n as the endogenous DNA primers.

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'

? For New Leaf Y screening, use the primers p-FMV05-5' and PVY02-3'.

? For secondary New Leaf Y verification, use pVY01-5' and pVY01-3'.

(3) Result analysis and decision

If PCR products of 216bp are detected in the solution containing the SSIIb-1 endogenous DNA primer and PCR products of 234bp and/or 225bp are detected in the solution containing the screening primer for New Leaf Plus and/or New Leaf Y, conduct PCR again on the same sample with the New Leaf Plus and/or New Leaf Y verification primer. If PCR products of 172bp or 161bp are confirmed, the decision "New Leaf Plus and/or New Leaf Y detected" shall be given to the sample. If the decision "New Leaf Plus and/or New Leaf Y detected" is given to any of the two DNA samples, the final decision "New Leaf Plus and/or New Leaf Y detected" shall be given to the product in question. If PCR products derived from endogenous genes are not detected in one of the two DNA samples, conduct electrophoresis again to identify PCR products. If PCR products for endogenous genes are not detected even in the second analysis, the results from the solution shall be discarded and the decision shall be given based on the results from the other DNA solution. If PCR products for endogenous genes are detected in neither of the two DNA solutions, conduct DNA extraction and PCR again and make a decision. If endogenous gene PCR products are not detected in a third DNA solution, the decision "Not-testable" shall be given. An illustrative decision-making matrix is provided in Table 10.

Table 10. An illustrative decision-making matrix for StarLink corn (CBH351) and GM potato

Sample No.		1	2	3	4	5	6	7	8	9
Extract 1	Endogenous DNA	+	+	+	+	+	+	+	+	-
	Screening	+	+	+	+	-	-	+	+	/
	Verification	+	+	+	+	/	/	-	-	/
Extract 2	Endogenous DNA	+	+	+	-	+	-	+	-	-
	Screening	+	+	-	-	-	-	+	-	/
	Verification	+	-	/	/	/	/	-	/	/
Decision		Detected	Detected	Detected	Detected	Not-detected	Not-detected	Not-detected	Not-detected	/

* For Sample 9, DNA extraction is conducted a third time.
** +: detected; -: not-detected; and /: tests are not necessary.

Addenda

This notice shall go into effect upon announcement.