Feed derived from recombinant DNA technology

Section 1　Overview of feed derived from recombinant DNA technology

Inspection of feed derived from recombinant DNA technology is conducted with the objective of monitoring the rate of contamination with recombinant DNA or protein product of genetically modified organism which has not been evaluated its safety yet.

1　Current status of genetically modified crop cultivation

Cultivation of feed crop derived from recombinant DNA technology basically started in the United States and Canada around 1996 with the cropping of corn, soybean and rapeseed.　In the 12 years up to 2007, the acreage of genetically modified crops increased by more than 67 times, with approximately 80 % of the corn currently grown throughout the US, which are created to possess insect resistance, herbicide tolerance or other properties utilizing the recombinant DNA technology.

2　Confirmation of safety, etc.

The Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) initially stipulated requirements for safety confirmation of feed and feed additives derived from recombinant DNA technology within the vice-ministerial notices “Safety Assessment Guidelines for Feed Derived from Genetically Modified Organisms” and “Safety Assessment Guidelines for Feed Additives Derived from Genetically Modified Organisms” respectively on April 19th and May 17th, 1996.　Corresponding to the growing international concern for the safety of feed derived from recombinant DNA technology, compulsory confirmation of safety was specified within the April 1st, 2003 revision of “the Ministerial Ordinance Concerning the Ingredient Specifications for Feeds and Feed Additives” (Ordinance of Ministry of Agriculture and Forestry, No. 35, 1976), prohibiting the domestic distribution of unconfirmed crops as livestock feed and feed additives.　Reflecting the inevitable possibility that imported feed ingredients may be unintentionally contaminated with genetically modified varieties unapproved of domestically but already approved by foreign countries with established safety criteria equivalent to Japanese standards, acceptable contamination rate of less than 1 % was specified for genetically modified varieties whose safety is yet to be confirmed.　As of April 1st, 2013, 67 varieties of feed and 5 varieties of feed additives derived from recombinant DNA technology are approved as safe for use within Japan.

In order to implement the compulsory safety confirmation requirements, the method of inspection for feed derived from recombinant DNA technology was stipulated as “Method of Inspection for Feed Derived from Recombinant DNA Technology” (as described within Section 2), calling for the inspection of StarLink Corn inclusion utilizing either the lateral flow method or qualitative PCR Method.　Feed proved positive in the above inspection is subjected to quantitative inspection using ELISA method to determine the contamination rate of the StarLink variety.　Corresponding to the distribution of seed for cultivation contaminated with Bt10 and DAS59132 varieties within the US market, qualitative PCR inspection was adopted on May 20th, 2005 to detect the inclusion of Bt10, and furthermore, real-time PCR inspection was introduced on June 18th, 2008 to identify DAS59132 contamination.

In response to three previous instances of minute contamination with unconfirmed varieties of genetically modified crops imported from United States, the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) adopted additional risk management policies on December 25th, 2008, specifying the responsibilities of bio-technology corporations and import/export firms, as well as the criteria and requirements for the verification of validity of pre-shipment inspections and the termination thereof. Corresponding to the introduction of this risk management policy, imported crops which had been proven as negative by the validated pre-shipment inspections were as excluded from the monitoring inspection conducted by the Food and Agricultural Materials Inspection Center (FAMIC).

3 Contamination of feed by unapproved feed varieties derived from recombinant DNA technology and other issues

(1) StarLink (CBH351) corn incident (2000)

Food intended for human consumption produced in the United States was found to be contaminated with a variety of corn (CBH351, named StarLink) genetically engineered to produce an insecticidal protein (Cry9C). StarLink corn was considered environmentally safe and approved for the US market as livestock feed. Furthermore, the contamination was confirmed in food imported into Japan. The Japanese Ministry of Health, Labour and Welfare responded to this development with the revision of the Food Sanitation Act specifications to adopt compulsory safety confirmation and prohibited the distribution of StarLink corn within the domestic market.

Accordingly, the Ministry of Agriculture, Forestry and Fisheries arranged to have the corn exported from the US as feed inspected for StarLink contamination prior to shipment, concurrently implementing monitoring inspection of the contamination of imported corn by StarLink to ensure the safety of feed. The inspections at the time involved confirmation testing by lateral flow and qualitative PCR methods as well as the determination of the contamination rate of the insecticidal protein Cry9C utilizing ELISA test. In conducting qualitative PCR inspections, MAFF initially procured the detection primer required for the testing of the recombinant DNA from the firm that initially developed the StarLink variety while developing alternative DNA detection primers in cooperation with the National Food Research Institute and various other research institutes.

Details pertaining to the inspection conducted relating to the StarLink contamination incident are disclosed as a press release issued by the Animal Feed Division, Livestock Industry Department, Agricultural Production Bureau of the MAFF dated June 11th, 2003.

(2) Bt10 incident (2006)

Bt10, a corn variety which was genetically engineered to produce an insecticidal protein (Cry1Ab) and a herbicide-resistant protein (PAT) and to possess antibiotic-resistance gene, and which had not been approved within the United States, accidentally mingled with the seed of Bt11, a corn variety approved for public cultivation through human error. The cultivated area for the unapproved variety, Bt10 amounted to approximately 0.1 percent of the overall cultivated area of corn within the US for the duration of 2003 to 2006. The situation became open in March 2007, but the US government maintained the position that the Bt10 variety did not pose a health concern, making no move to recover the variety. The cultivation seed contaminated with Bt10 was subsequently withdrawn by Syngenta

Seeds, preventing the sowing of the variety from the year 2007 onward.

The Ministry of Agriculture, Forestry and Fisheries, (MAFF) notified the revision of "Method of Inspection for Feed Derived from Recombinant DNA Technology" based on the analysis method indicated by Syngenta Seeds, to conduct the inspection for corn imported from the United States.

Additionally, corn imported from the US was designated as feed suspected not to comply with the specifications as of August 31st, 2007, and to be reported prior to import in accordance with the provisions of the Feed Safety Law.

Regarding Bt10, the Food Safety Commission submitted a food safety risk assessment report to the MAFF dated June 15th 2006. Within the report, the Commission concluded that "the safety evaluation of the Bt10 variety was difficult, and the health risk to humans cannot be determined at the current stage". Accordingly, the MAFF decided not to implement a safety confirmation of the Bt10 variety for use as feed.

As a result of the inspection conducted as of October 2006, Bt10 contamination was identified in 16 instances from approximately 42,000 tons of imported corn, but has not been detected since then.

(3) DAS59132 incident (2008)

A corn variety DAS59132, which was genetically modified to produce an insecticidal protein (Cry34/35Ab1) and herbicide-resistant protein (PAT), and which had not been approved within the United States accidentally mingled with the seed of DAS59122, a corn variety approved for public cultivation through human error, to be sown for the duration of 2006 to 2007. The cultivated area for the unapproved variety DAS59132 amounted to approximately 0.06 percent of the cultivated area of corn within the US for 2007, with a contamination rate presumed to be less than 0.0002 percent of the overall US corn harvest of 2007. Both US and Japanese governments maintained positions that the DAS59132 contamination did not pose a health concern, and made no attempts to recover the variety. The cultivation seed contaminated with DAS59132 was subsequently withdrawn by Dow AgroScience, preventing the sowing of the variety from the year 2008 onward.

The MAFF notified the revision of the "Method of Inspection for Feed Derived from Recombinant DNA Technology" based on the analysis method indicated by Dow AgroScience, to conduct the inspection for corn imported from the United States.

As a result of the inspection conducted, no examples of DAS59132 contamination were detected from the corn imported from the US.

Section 2　Method of Inspection for Feed Derived from Recombinant DNA Technology

In this section, the contents of "Method of Inspection for Feed Derived from Recombinant DNA Technology" (attachment 3 of ministerial notice No. 8598 dated April 1st, 2003 by director general of Production Bureau, Ministry of Agriculture, Forestry and Fisheries) is recompiled providing notes and precautions for sections denoted within the notice with "*" symbols. However, Notes and precautions marked with the letter "A" are addendums provided alternately by the compiler when deemed necessary.

Distinctive errors in the text of the notice and passages potentially misleading in the implementation of the operations analysis are rephrased, with the modification indicated within the relevant passage.

[Cautions for conducting inspections of feed derived from recombinant DNA technology]

Samples shall be prepared using ultracentrifugal mills by pulverizing to fineness enabling sample to pass through a 0.5 mm sieve.

Workspace shall be cleansed by atomizing with 70 % ethanol solution and wiping surfaces using paper towels, etc. prior to analysis. To prevent contamination by scattering of ground product, conduct analysis with the surroundings covered using plastic wrap or sheets. Conduct analysis wearing disposable gloves after cleansing all equipment involved in the analytical process by atomizing with 70 % ethanol solution and wiping surfaces using paper towels, etc. Surface-coverings (wraps/sheets), gloves and medicine spoons, etc. shall be replaced for each sample.

[Content of Ministerial Notice]

1　Sampling method [Method of Inspection for Feed Derived from Recombinant DNA Technology　1]

1.1.　Sampling for inspection of feed derived from recombinant DNA technology

1.1.1.　Sampling of corn kernel

Based on the assumption that the corn kernel derived from recombinant DNA technology has a heterogeneous distribution, the following method of sampling shall be employed depending on the lot size and packaging types to enable samples representative of the subject lot to be obtained. In obtaining samples, adequate considerations are required in preventing the inclusion of kernels from other lots. Either use disposable instruments, containers and packaging, or sufficiently cleanse in case of reuse.

Mix obtained sample to homogenize the obtained kernels, then collect a certain quantity[1] required for the inspection and pulverize homogeneously using grinders, etc.

1.1.1.1. In case of feed encased in sacks:

Samples shall be collected as indicated in the table below:

Lot size			Unpacking quantity for sampling	Sampling quantity (kg)	Number of samples
	≦	15	2	1.5	1
16	~	25	3	1.5	1
26	~	90	5	1.5	1
91	~	150	8	1.5	1
151	~	280	13	1.5	1
281	~	500	20	1.5	1
501	~	1,200	32	1.5	1
1,201	~	3,200	50	1.5	1
3,201	~	10,000	80	1.5	1
10,001	~	35,000	125	1.5	1
35,001	~	150,000	200	1.5	1
150,001	~	500,000	315	1.5	1
	≧	500,001	500	1.5	1

1.1.1.2. In case of feed stocked in bulk:

1.1.1.2.1. During silo installation

While installing corn in silos, collect samples with automatic samplers, etc. to enable the collected samples to represent the entire lot consisting of the contents of a single silo. Collect a minimum quantity of 10 kilograms in 15 sessions respectively set at adequate intervals, conducting sample reduction of the total amount to obtain samples (respectively more than 1.5 kilograms) per silo.

For corn already installed in silos, sampling shall be conducted in a likewise manner when transferring the contents to alternate silos.

1.1.1.2.2. During barge installation:

While loading feed onto barges (including domestic vessels), collect samples with automatic samplers, etc. to enable the collected samples to represent the entire lot consisting of the contents of a single barge. Collect a minimum quantity of 10 kilograms in 15 sessions respectively set at adequate intervals, conducting sample reduction of the total amount to obtain samples (respectively more than 1.5 kilograms) per barge.

«Notes and precautions»

[1] The qualitative and quantitative analysis of CBH351 corn shall be conducted using 2400 kernels, while the qualitative analysis of Bt10 corn and DAS59132 corn shall be performed respectively with 500 grams of kernels.

2 Analytical methods for CBH351 corn [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.]

2.1 Qualitative analysis of CBH351 corn [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.1.]

Inspection to be conducted by Lateral Flow or Qualitative PCR method using corn kernels.

2.1.1 Preparation of the sample[A1] [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.1.1.]

Kernels to be subjected to the analysis shall be perfect kernels obtained upon elimination of crushed grains and miscellaneous contaminants. Samples shall be prepared by adequate cleansing and desiccation[A2], confirming the surface of the kernels are not contaminated by incrustation.

It is recommended that the ground samples subjected to the lateral flow or qualitative PCR analysis shall be processed to a fineness enabling the sample to pass through 0.5 mm sieve[A3]. Adequate care is required in pulverizing process and the handling of instruments involved in the inspection to prevent the contamination among respective samples. For measures for contamination prevention, refer to "Japanese Agricultural Standard (JAS) analytical test handbook: Genetically modified food quality, labeling analysis manual for individual products (revision 3) VI. Prevention of Contamination"[A4].

«Notes and precautions»

[A1] For notes on conducting an analysis, refer to "Cautions for conducting inspections for feed derived from recombinant DNA technology".

[A2] Remove contaminants adhered to the surface of the perfect corn kernels to be used in the analysis by washing with tap water, rinsing the sample adequately with distilled water. Dry sample sufficiently at room temperature in a contamination-free environment such as a clean-bench. Confirm sample to be of adequate quantity (weight) to conduct an analysis (2400 kernels or 500 grams) and place in frozen storage at the temperature of −20 °C in a clean container until the pulverization.

[A3] Corn samples may be frozen in liquid nitrogen prior to preparation with the grinder to prevent the degeneration of sample due to the heat of pulverization. Pulverized samples shall also be placed in frozen storage at the temperature of −20 °C in a clean container until the analysis.

[A4] Japanese version of "Japanese Agricultural Standard (JAS) analytical test handbook: Genetically modified food quality, labeling analysis manual for individual products (revision 3)" is available for download from the website of Food and Agricultural Materials Inspection Center (FAMIC).

2.1.2 Lateral flow method [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.1.2.]

2400 kernels of corn shall be subjected to the lateral flow tests, to be tested in sessions according to the specifications of the test kit, 3 sessions for an 800-kernel kit or 4 sessions for a 600-kernel kit as the case may be. Commercially available test kits used in the test shall be capable of achieving test results equivalent to TraitChek™ Bt9 Grain Kit, 100 Strips (Part# 7000012) manufactured by Strategic Diagnostics (SDI) or Reveal® for Cry9C Strip Test (Part# 8003) from Neogen Corporation. The test methods described below basically conforms to the specifications of the manuals provided with respective kits. In the event the specifications or procedures for the use of the test kit is altered, the tests shall be conducted compliant to the descriptions provided within the test kit manual. Unless otherwise indicated, it is recommended that the water used in the test be processed by reverse osmosis membrane purification (RO water) or distilled water.

2.1.1.2.1. TraitChek™ Bt9 Grain Kit, 100 Strips (Part# 7000012)

2.1.1.2.1.1. Experimentation procedure

From the sample corn kernels collected, randomly select 800 perfect kernels for pulverization, place the ground product in a wide-mouthed container with a capacity of about 500 mL. Add 250 mL of water and shake the container for 10 to 20 seconds until the sample is completely moistened. Allow content to settle. If supernatant solution is not produced in this condition, add small amounts of water and repeat process until the supernatant solution is produced. Then transfer 0.5 mL of supernatant solution to a 1.5 mL capacity tube included in the kit and perpendicularly place a Trait Bt9 test strip into the tube[A1].

2.1.1.2.1.2. Judgment

Observe indicator of the test strip five minutes after inserting the strip into the tube[1]. The sample is judged to be positive if the test strip displays two red lines, and negative if the test strip displays only one control line. The test is considered invalid if no line is displayed, and calls for retesting. In the event any of the 3 sessions conducted on a total of 2400 kernels returns a positive result, the sample is deemed positive, and shall be subjected to quantitative analysis.

2.1.1.2.2. Reveal® for Cry9C Strip Test (Part# 8003)

2.1.1.2.2.1. Experimentation procedure

From the sample corn kernels collected, randomly select 800 perfect kernels for pulverization, place the ground product in a wide-mouthed container with a capacity of about 500 mL. Add 400 ± 20 mL of water and shake the container for 30 to 40 seconds until the sample is completely moistened. Allow content to settle. Then transfer 0.5 mL of supernatant solution to a 1.5 mL capacity tube included in the kit and perpendicularly place a Cry9C test strip into the tube[A1].

2.1.1.2.2.2. Judgment

Observe indicator of the test strip ten minutes after inserting the strip into the tube[2]. The sample is judged to be positive if the sets strip displays two red lines, and negative if the test strip displays only one control line. The test is considered invalid if no line is displayed, and calls for retesting.

In the event any of the 3 sessions conducted on a total of 2400 kernels returns a positive result, the sample is deemed positive, and shall be subjected to quantitative analysis.

«Summary of the analysis method»

The following is a description of the method of detecting Cry9C protein using lateral flow inspection:

Flowsheet for the analysis is indicated in figure 17.1-1 below:

Pulverization (2400 kernels)
|
Extraction (800 kernels × 3 sessions or 600 kernels × 4 sessions)
|
Transfer supernatant solution into 1.5 mL tube after allowing extract to settle
|
Place a test strip into the tube and allow strip to react for specified duration of time
|
Judgment

Figure 17.1-1 Flowsheet for detection of Cry9C protein by Lateral Flow inspection

Reference Ministry of Health, Labour and Welfare: Inspection method of food derived from recombinant DNA technology

Food and Agricultural Materials Inspection Center (I.A.A.): Japanese Agricultural Standard (JAS) analytical test handbook: Genetically modified food quality, labeling analysis manual for individual products

«Method validation»

Limit of detection: Approximately 0.125 % as contamination rate.

«Notes and precautions»

[1] Red indicator line may get dark if the strip is allowed to react for more than five minutes, thereby obstructing accurate judgment.

[2] Red indicator line may get dark if the strip is allowed to react for more than ten minutes, thereby obstructing accurate judgment.

[A1] Use tweezers when handling test strips and avoid touching strips with bare hands. Take out only the quantity necessary for testing, and store the remaining strips according to storage instructions provided within the test kit.

2.1.3 Qualitative PCR analysis [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.1.3.]

Qualitative PCR is a method to detect segments of extracted DNA by electrophoresis upon amplification of the DNA using primers.

2.1.1.3.1. DNA extraction and purification from corn kernels

Extraction of the corn DNA is performed using 2400 perfect kernels randomly selected from among the obtained samples. The kernels are to be pulverized homogeneously, then subjected to DNA extraction using such means as silica-spin-columns or using a surfactant agent such as cetyltrimethyl ammonium bromide (CTAB). In view of the procedural ease, extraction method using the silica-spin-column is recommended. In the event alternative methods are to be employed, the extraction of DNA suitable for PCR amplification shall be confirmed. In this section, the procedures for DNA extraction utilizing the silica-spin-column method are described.

Unless otherwise indicated, the water used in DNA extraction shall be ultra pure water over 17.0 MΩ·cm processed by using ultra pure water production system from reverse osmosis (RO) processed water or distilled water. The ultra pure water shall then be autoclaved for at least 15 minutes at 121 °C. Disposable tubes and chips shall be utilized for the extraction, also to be sterilized for 15 minutes with autoclaves at 121 °C or treated with gamma radiation (filtered-tips are recommended for use in combination with micropipettes).

Duplicate sequences of DNA extraction shall be performed for respective samples.

2.1.1.3.1.1. Extraction method using silica-spin-column kits (for DNeasy® Plant Maxi Kit manufactured by QIAGEN)

Measure and transfer 1.0 g of homogeneously pulverized sample into a 15 mL capacity tube, add 5 mL of API buffer[A1] preheated to 65 °C and 10 μL of RNase A (included in the kit), and mix by overturning tube until traces of powdered sample residue are removed from the bottom of the tube, and mix well using a vortex tube mixer. Incubate in water bath maintained at 65 °C for one hour. During the incubation period, overturn-agitate sample three times at 15 minute intervals, then agitate tube for 10 seconds with the vortex tube mixer set to maximum. After the reaction, add 1.8 mL of AP2 buffer to the tube, agitate for 10 seconds with the vortex tube mixer set to maximum and cool in ice-cold water for 15 minutes[A2]. Centrifuge the sample for 15 minutes in room temperature at 3000×*g* [1][A3]. Collect 4.2 mL of supernatant solution[2], load onto the QIAshredder™ Maxi spin column (lilac) and centrifuge again for 5 minutes in room temperature at 3000×*g* [1] and transfer 4 mL of supernatant solution to a new 50 mL-capacity tube [2]. The tube shall then be agitated for 10 seconds on the vortex tube mixer set to maximum speed. Collect 3.4 mL of the sample and transfer to a new 50 mL-capacity tube. Add 5.1 mL of adjusted AP3/Et-OH buffer[A4] and agitate for 10 seconds on the vortex tube mixer set to maximum speed. Load the entire solution onto the DNeasy® spin column (colorless) and centrifuge for 5 minutes in room temperature at 3000×*g* [1]. Discard the eluate and load 12 mL of adjusted AW buffer onto the column. Centrifuge the sample for 15 minutes in room temperature at 3000×*g* [1]. Transfer the column to a Collection tube (50 mL)

included in the kit, add 1 mL of water preheated to 65 °C and allow to settle for 5 minutes in room temperature. Centrifuge the tube for 10 minutes in room temperature at 3000×*g* [1]. Measure the quantity of the eluate obtained in the process[A5], and transfer to a 2 mL-tube. Add isopropanol equivalent in amount to the eluate[A6]. Slowly mix solution by overturning tube 10 times, and allow to settle in room temperature for 5 minutes. Centrifuge tube for 15 minutes at 4 °C, 12000×*g*. Discard supernatant solution. Add 500 μL of ethanol diluted to 70 %[A6] and wash away precipitate[A7], then subject to centrifugation for 3 minutes at 4 °C, 12000×*g*. Discard supernatant solution. Allow precipitate to dry[A8], add 50 to 100 μL of TE buffer[3] to dissolve precipitate[A9]. The resulting solution shall be used as the undiluted DNA sample.

2.1.1.3.2. Confirmation of the purity of undiluted DNA sample, preparation and storage of DNA sample solution

Take an appropriate amount of the undiluted DNA sample and dilute it 10 to 50 times by adding TE buffer for analysis using the spectrophotometer[A10]. Measure the UV absorption in the range of 200 to 300 nm to acquire the absorbance in 230 nm, 260 nm, and 280 nm (O.D. 230, O.D. 260, and O.D. 280[4]). If the ratio of O.D. 260/O.D. 280 is within the range of 1.7 to 2.0, the DNA is sufficiently purified. DNA concentration for the undiluted sample is then calculated upon an assumption that the DNA concentration when O.D. 260 = 1 is 50 ng/μL. Based on the calculated value, prepare a 10 ng/μL DNA solution for PCR analysis, adjusting the concentration by diluting with water if necessary[A11]. Dispense solution into micro-tube to be cryopreserved at below −20 °C. Dispensed DNA solutions shall be used in the analysis immediately upon thawing[A12]. In the case that the DNA concentration within the undiluted sample is lower than the specified concentration for the PCR analysis, the undiluted sample shall be used without adjustment.

2.1.1.3.3. PCR amplification

In polymerase chain reaction (PCR), even trace quantities of template DNA are amplified. Accordingly, it is necessary to take extensive care not to allow the contamination of the sample with foreign DNA (especially PCR amplification products). As DNA can be decomposed by DNA decomposition enzyme (DNase) secreted from the surface of the human skin, contamination from the enzyme must also be prevented. It is therefore necessary to use disposable tubes and chips, to be sterilized for 15 minutes within autoclaves set to 121 °C or treated with gamma radiation (filtered-tips are recommended for use in combination with micropipettes).

Unless otherwise indicated, the water used in DNA extraction shall be ultra pure water over 17.0 MΩ·cm processed by using ultra pure water production system from reverse osmosis (RO) processed water or distilled water. The ultra pure water shall then be autoclaved for at least 15 minutes at 121 °C.

For the procedure, dedicated rubber gloves shall be worn[A13], with the reagents and tubes placed on an ice surface.

2.1.1.3.3.1. Preparation of the reaction solution for PCR

Reaction solution for PCR shall be prepared for respective reaction tubes as indicated below. With

the reaction tube containing a solution consisting of PCR buffer, 0.20 mmol/L dNTP solution[5], 3.0 mmol/L of magnesium chloride solution[A14], 0.2 μmol/L solutions respectively of 5′ and 3′ primers[6][A15], and 0.625 units of DNA polymerase[7][A16] placed on ice surface, add 2.5 μL of sample DNA solution pre-adjusted to 10 ng/μL concentration (equivalent to 25 ng of DNA) to obtain an overall quantity of 25 μL[A17] of reaction solution. The reaction tube is then set onto the PCR thermal cycler[8]. Conditions for the reaction are as indicated below: Maintain 95 °C for 10 minutes to initiate the reaction, then allow the PCR amplification to proceed for 40 cycles with 95 °C for 0.5 minute, 60 °C for 0.5 minute and 72 °C for 0.5 minute as one reaction cycle. After 40 cycles, maintain 72 °C for 7 minutes and store sample at 4 °C. The end result will be used as the PCR amplification product solution. Concurrently to the preparation of the reaction solution for PCR, a solution without primers and a solution not containing the DNA sample solution shall also be prepared for use as negative controls to the PCR reaction. Commercially available plasmids[6][A18] shall be provided as positive controls for the analysis. In order to confirm the extraction of DNA from the collected samples, PCR amplification using primers corresponding to the endogenous genes of corn shall be performed for respective sample DNA solutions[A19].

2.1.1.3.4. Agarose gel electrophoresis

Isolate PCR amplification product by agarose gel electrophoresis to confirm the amplified DNA bands.

2.1.1.3.4.1. Preparation of the agarose gel

Weigh agarose as to make the gel concentration to 3 %[A20], add TAE buffer[9][A21] and heat to allow agarose to dissolve. When prestaining gel, add 5 μL of ethidium bromide solution for every 100 mL of dissolved gel (10 mg/mL)[10][A22]. After gel has cooled to about 50 °C, inpour into the gel maker and attach a comb. Allow gel to cool to room temperature and remove the comb from the solidified gel. Although it is desirable to use the gel immediately upon preparation, it may be preserved for several days by immersing in buffer liquid.

2.1.1.3.4.2. Electrophoresis

Set gel within electrophoresis tank[A23] containing TAE buffer. Mix 7.5 μL of PCR amplification product solution and appropriate quantities of gel loading buffer[A24], then pour into the gel well after mixing. Allow DNA ladders[A25] to run in the same well. Samples shall be injected into the gel promptly as the DNA diffuses if the injection takes too long, rendering it difficult to obtain distinct results. Then apply 100 V rated voltage to initiate electrophoresis, terminating process when the BPB solution contained in the gel loading buffer infiltrates 1/2 to 2/3 of the gel.

2.1.1.3.4.3. Poststaining of gel

The following procedure is not required in case the prestaining of the gel is applied.

In a container filled with TAE buffer in quantities capable of immersing the gel, transfer the gel subjected to electrophoresis. Add 5 μL of ethidium bromide solution for every 100 mL of buffer liquid and stain for 30 minutes while lightly shaking the container on a shaker.

2.1.1.3.4.4. Gel image analysis

Place food packaging wrap[11] on the stage within the gel image analyzer, put stained gel on the wrap and irradiate with ultraviolet rays (312 nm)[12], and confirm image of the electrophoresis pattern displayed by the analyzer. Compare image to the DNA ladders as well as the positive control to determine the presence (or absence) of the relevant bands of PCR amplification product. In the event PCR amplification product bands corresponding to negative control is detected, the results subsequent to the DNA extraction process is deemed invalid, and must be re-implemented. Results of the electrophoresis shall be saved as image data.

2.1.1.3.5. Judgment

In the event the presence of 157 bp PCR amplification product corresponding to endogenous gene primers (as control primers), as well as 170 bp PCR amplification product corresponding to CBH351 detection primers and 171 bp PCR amplification product corresponding to CBH351 confirmation primers are all identified within either of the dually extracted samples as a result of the electrophoresis, the sample is deemed CBH351 positive.

When both extracted samples indicated the presence of the band of PCR amplification product of the control primers, but failed to indicate the band of PCR amplification product of the CBH351 identification primers, the sample is deemed CBH351 negative. Even if the sample indicated the band of the CBH351 identification primers, it is deemed CBH351 negative in the event it failed to indicate the band of the CBH351 confirmation primers.

In the event either of the sample fails to indicate the presence of endogenous genes for the control primers, re-implement procedures from electrophoresis onward. If results do not change upon re-implementation, the DNA extraction sample shall be deemed invalid, and judgment shall be based on the results for the remaining sample. In case neither of the samples fail to indicate the presence of endogenous genes for the control primers, re-extract DNA and repeat the analysis.

Samples deemed CBH351 positive shall be subjected to quantitative analysis.

Examples of judgment

	Sample number	1	2	3	4	5	6	7	8	9
Extraction 1	Control primer	＋	＋	＋	＋	＋	＋	＋	＋	－
	Detection primer	＋	＋	＋	＋	－	－	＋	＋	／
	Confirmation primer	＋	＋	＋	＋	／	／	－	－	／
Extraction 2	Control primer	＋	＋	＋	－	＋	－	＋	－	－
	Detection primer	＋	＋	－	－	－	－	＋	－	／
	Confirmation primer	＋	－	／	／	／	／	－	／	／
Judgment		Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	／

Third DNA extraction process to be conducted in the case of sample number 9.

＋: positive, －: negative, and ／ : Test for confirmation is not required.

«Summary of the analysis method»

This method is to detect CBH351 genes using PCR analysis:

Flowsheet for the analysis is indicated in figure 17.1-2 below:

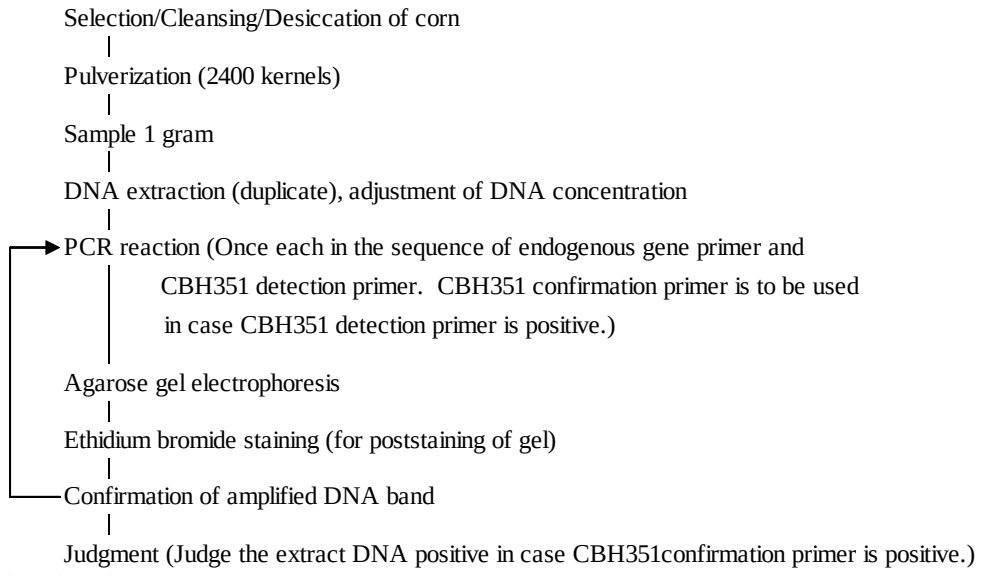


Figure 17.1-2 Flowsheet for detection of CBH351 genes by qualitative PCR

Reference Ministry of Health, Labour and Welfare: Notification of "Inspection method for food derived from recombinant DNA technology"

Food and Agricultural Materials Inspection Center: Japanese Agricultural Standard (JAS) analytical test handbook: Genetically modified food quality, labeling analysis manual for individual products

«Method validation»

Limit of detection: Approximately 0.05 % as contamination rate.

«Notes and precautions»

[1] Use a swing rotor for centrifugation procedure of the column.

[2] Avoid taking up the precipitate or upper layer membrane of the supernatant.

[3] TE buffer to be adjusted with water to the final concentration of 10 mmol/L for Tris- hydrochloric acid (pH 8.0) and 1 mmol/L for EDTA (pH 8.0).

[4] O.D. 230 considered to be the absorption minimum of DNA, O.D. 260 to be the absorption maximum of DNA, O.D. 280 regarded as absorption of impurities such as protein in the sample.

[5] PCR buffer: Use PCR buffer II (Applied Biosystems) or equivalent product.

[6] Primers and positive controls used in the analysis shall be as indicated below:

· GM Maize Detection Zein (for endogeneous gene) Oligonucleotide: Nippon Gene (#318-05671) or FASMAC (#M9-1M)

· GM Maize Detection CBH351 (for 1st screening) Oligonucleotide: Nippon Gene (#315-05681) or FASMAC (#M7-1M)

· GM Maize Detection CBH351 (for 2nd screening) Oligonucleotide: Nippon Gene (#312-05691) or FASMAC (#M8-1M)

· GM Maize Detection CBH351 Positive Control Plasmid: Nippon Gene (#317-04921) or FASMAC (#PM-3)

The reagents may be procured respectively from Nippon Gene Co., Ltd. (zip: 930-0983 1-8-7, Toiyamachi, Toyama-Shi; Tel. +81 76 451 6548, Fax. +81 76 451 6547) and FASMAC (zip: 243-0041 5-1-3 Midori-ga-Oka, Atsugi-Shi; Tel. +81 46 295 8787, Fax. +81 46 294 3738)

[7] DNA polymerase: Use AmpliTaq Gold® DNA polymerase (Applied Biosystems).

[8] PCR thermal cycler: Use GeneAmp® PCR System 9700 (Applied Biosystems) or equivalent apparatus.

[9] TAE buffer to be adjusted with distilled water to the final concentration of 40 mmol/L for Tris-acetic acid, 1 mmol/L for EDTA.

[10] Ethidium bromide is fluorescent reagent capable of intruding the gap between double strands of the DNA, and is a strong mutagen. Gloves must be worn when handling, as well as masks when handling in powder form.

[11] Food packaging wrap

Ultraviolet rays may be absorbed by the material resulting in the loss of image unless film made of polyvinylidene chloride is used.

[12] Ultraviolet (UV) rays

Ultraviolet rays are hazardous especially to the eyes. Take necessary precautions including the use of protective goggles.

[A1] AP1 buffer to be preheated in water bath at 65 °C.

[A2] The use of ice-cold water is indicated within this document for better results although the Notice specifies immersion in water.

[A3] Insufficient centrifugation may cause the solution to become turbid or flotage to occur, leading to decline in DNA amplification. Continue with the centrifugation until the supernatant solution achieves clarity.

[A4] Add guaranteed-grade ethanol to the buffer in quantities specified by the test kit prior to use.

[A5] Liquid quantity to be measured with micropipette when collecting.

[A6] Use guaranteed-grade reagents.

[A7] Tap on tube lightly with a fingertip to float the precipitate and wash the surface.

[A8] Use either a centrifugal evaporator or a small desiccator.

[A9] Adjust quantity of TE solution corresponding to the amount of precipitate.

Tap on tube added with TE solution with a fingertip and centrifuge for a short duration. This procedure shall be repeated several times. If insoluble substance can be confirmed visually, allow to settle for 12 to 24 hours within cool storage. Visually confirm absence of insoluble substance prior to use as DNA extraction solution. If insoluble substance is still present after 24 hours, centrifuge solution at 12000×*g* for 3 minutes in 4 °C environment, transfer the resulting supernatant solution to a new tube for use in DNA solution. Residual precipitate shall be kept in storage at −20 °C.

[A10] Quantity of sample solution to be used in measurement shall be adjusted corresponding to the capacity of the measurement cell incorporated in the spectrophotometer. For spectrophotometers capable of measurement with trace amounts of undiluted DNA samples, the dilution process is not necessarily required.

[A11] The water, chip and tube to be used in the preparation of the DNA solution shall be kept sealed

after sterilization until use. DNA solutions shall be prepared in quantities of 30 to 50 μL. When measured in solution diluted by 50 times, the DNA concentration in case O.D. 260 indicates value of 0.12 is calculated as follows:

DNA solution (χ): 50 (ng/μL) = 0.12 : 1, χ = 6 (ng/μL)

Above value is for solutions diluted 50 times, and accordingly, the concentration in the actual DNA solution shall be: 6 (ng/μL) × 50 = 300 (ng/μL)

In order to prepare 30 μL of the required 10 ng/μL DNA solution at this concentration, the content shall be adjusted to:

$$\frac{10\,\text{ng/μg} \times 30\,\mu\text{L}}{300\,\text{ng/μg}} = 1.0\,\mu\text{L}$$ DNA solution: 1.0 μL, sterilized water: 29.0 μL

[A12] DNA degrades when subjected to repeated freezing/thawing. It is therefore desirable to dispense solutions into small quantities for usability. Allow solution to thaw gradually on an ice-surface or within cool storage chambers.

[A13] Use disposable gloves.

[A14] Use reagents included with the procured DNA polymerase. Prepare solution containing specified concentrations of PCR reaction buffer, magnesium chloride and dNTP, to be dispensed into small quantities and kept in frozen storage at −20 °C for usability. To prevent contamination, pre-mixed solutions kept in frozen storage shall be discarded after thawing for use.

[A15] Primers degrade when subjected to repeated freezing/thawing. It is therefore desirable to dispense solutions into small quantities in tubes to be kept in frozen storage upon opening seal for usability.

[A16] Acute mixing causes reagents to degrade. Mixing shall be conducted gently by pipetting.

[A17] In preparing samples, set the necessary quantity of tubes (number of samples + three for controls) onto a tube rack, to be placed on an ice-surface throughout the process. Mixing quantity of contents to obtain 25 μL of PCR reaction solution for CBH351 inspection is as indicated in a table below. Reagents other than the DNA sample solution shall be premixed in alternate tubes, to be dispensed into the reaction tubes for the purpose of usability. In this situation, one extra tube of solution shall be prepared (necessary quantity + 1). Example of preparations required to provide for the analysis of 4 samples and associated controls are indicated in the following:

Water: 13.975 μL × 8 = 111.8 μL; 10xPCR buffer II: 2.5 μL × 8 = 20 μL; magnesium chloride (25 mmol/L): 3.0 μL × 8 = 24 μL; dNTP (2.0 mmol/L): 2.5 μL × 8 = 20 μL; and enzyme (5 unit/μL): 0.125 μL × 8 = 1.0 μL to be mixed sufficiently within 1.5 mL-capacity tube. 22.1 μL of the mixed liquid is then dispensed into a PCR tube not containing primers, adding the primers and equivalent quantity of water (0.4 μL) to obtain 22.5 μL of solution. Add primers to remaining mixed liquid: 0.4 μL × 7 = 2.8 μL and dispense 22.5 μL of the liquid to each of the PCR reaction tubes. Add 2.5 μL of the DNA sample solutions for the tubes to obtain a total content of 25 μL per tube. The addition of the sample solution shall be conducted in the sequence of DNA sample solutions, negative control and positive control. DNA sample solutions to be added to the primer-less and positive control solutions shall be positive control plasmid instead of the sample DNA. Content of the tube to be prepared without the DNA sample solution shall be adjusted by adding 2.5 μL of water.

Reagent	Quantity
10xPCR buffer II	2.5 µL
Magnesium chloride (25 mmol/L)	3.0 µL
dNTP (2.0 mmol/L)	2.5 µL
Enzyme (5 unit/µL)	0.125 µL
Primer mix (25 µmol/L)	0.4 µL
Water	13.975 µL
DNA sample solution (10 ng/µL)	2.5 µL

[A18] To prevent contamination, avoid handling positive control plasmids concurrently with the analysis samples.

[A19] After completing the reaction, store solution in cold or frozen storage, or immediately proceed to the electrophoresis procedure.

[A20] Agarose gel shall be Agarose L03 "TAKARA" (Takara Bio Inc.) or equivalent.

[A21] As large quantities of TAE buffer is required for the production of gel and the electrophoresis procedure, 50x concentrate of the solution shall be prepared, to be 50-fold diluted as needed. To prepare 50xTAE solution (1000 mL), mix 242.3 g of Tris, 100 mL of 0.5 mol/L EDTA (pH8.0) and 57.1 mL of acetic acid, then adjust to 1000 mL by adding water.

[A22] Used ethidium bromide solution to be adequately decomposed by using commercially available disposal instruments.

[A23] Electrophoresis to be performed using Mupid II electrophoresis equipment (manufactured by Advance Co. Ltd.) or equivalent.

[A24] Buffer solutions often include two types of coloring agents, xylene cyanol and bromophenol blue (BPB). Bromophenol blue is the agent mobilized ahead of DNA amplification product during the electrophoresis process. Coloring agents included with DNA ladders may be used.

[A25] 100 bp DNA ladders are appropriate.

3 Quantitative analysis of CBH351 corn [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.2.]

Quantitative analysis is conducted by means of ELISA assay of corn kernels.

3.1 Preparation of samples [A1] [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.2.1.]

Kernels to be subjected to the inspection shall be perfect kernels obtained upon elimination of crushed grains and miscellaneous contaminants. Samples shall be prepared by adequate cleansing and desiccation[A2], confirming the surface of the kernels is not contaminated by incrustation. Analysis is performed using 2400 perfect kernels randomly selected from among the obtained samples, to be pulverized homogeneously.

Quantitative values derived from the ELISA assay is influenced by the particle size of the pulverized sample. Accordingly, the pulverization shall be performed in compliance with the method specified by the test kit. To prevent cross-contamination between the samples, adequate precaution shall be taken pertaining to the environment and the handling of equipment during the pulverization process. For contamination prevention measures, refer to "Japanese Agricultural Standard (JAS) analytical test handbook: Genetically modified food quality, labeling analysis manual for individual products (revision 3) VI. Prevention of Contamination".

3.2 ELISA assay [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.1.2.2.]

Use commercially available test kits, namely GMOChek™ Bt9 Maize Test Kit (Part# 7110030) manufactured by Strategic Diagnostics (SDI) or equivalent products. The test method described below basically corresponds with the specifications in the manual provided with the kit. In the event the specifications or procedures for the use of the test kit is altered, the tests shall be conducted in compliance with the descriptions provided by the test kit manual. Unless otherwise indicated, it is recommended that the water utilized in the test be reverse osmosis (RO) processed water or distilled water.

2.1.2.2.1. Preparation

All reagents shall be removed from cold storage 1 hour prior to use in order to restore to room temperature. The necessary quantity of the diluted buffer solution shall be prepared beforehand, corresponding to the number of the samples to be analyzed. Dual instances of analysis shall be performed for each sample, with the average of the two results adopted as the quantitative value.

2.1.2.2.2. Experimental procedure

Weigh out 4.0 grams of homogeneously pulverized ground products into an appropriate container, add 48 mL of diluted buffer solution and shake for 1 minute using a shaker or vortex tube mixer. Perform three sessions of 1-minute shaking process at 10 minute intervals[A3], then transfer 15 mL of supernatant solution to a centrifuging tube. Centrifuge tube at 3000 to 5000 rpm for 5 minutes and use resulting supernatant solution as sample solution.

Drop 100 μL of Bt9 enzyme complex into each of the wells on the plate[A4], and likewise drop 100 μL of standard and sample extract[1][A5] solutions. Cover wells with plate sealer and mix sufficiently for approximately 30 seconds. Allow solutions to react for 1 hour at room temperature upon shading. After the reaction is complete, cleanse sample using 300 μL of the diluted buffer solution. The sequence shall be repeated for five times. Do not allow droplets to remain in the wells after cleansing. Once the cleansing procedure is completed, drop 100 μL of chromogenic substrate solution, mix well for 30 seconds, and allow solution to react for 10 minutes at room temperature upon shading. After the reaction period, drop 100 μL of reaction stop solution into each of the wells to terminate the reaction. Absorbance of the sample solutions is to be measured using a micro-plate photometer (450 nm) within 15 minutes of the dropping of the reaction stop solution.

2.1.2.2.3. Test acceptance criteria

Results from the test are deemed acceptable when the following criteria are satisfied:

(a) Mean of the absorbance for negative control is less than 0.20.

(b) Mean of the absorbance for the 0.10 % standard is more than 1.0.

(c) Coefficients of variation (CV) for the 0.0075 %, 0.025 % and 0.10 % standards are less than 15 % respectively.

(d) Correlation coefficient of the linear regression of the standard sample is more than 0.96 and the correlation coefficient in case of polynomial is more than 0.98.

(e) Repeated measurement of absorbance for respective sample indicates a CV of less than 15 % in case the mean of the absorbance in the repeated measurement is higher than 0.20.

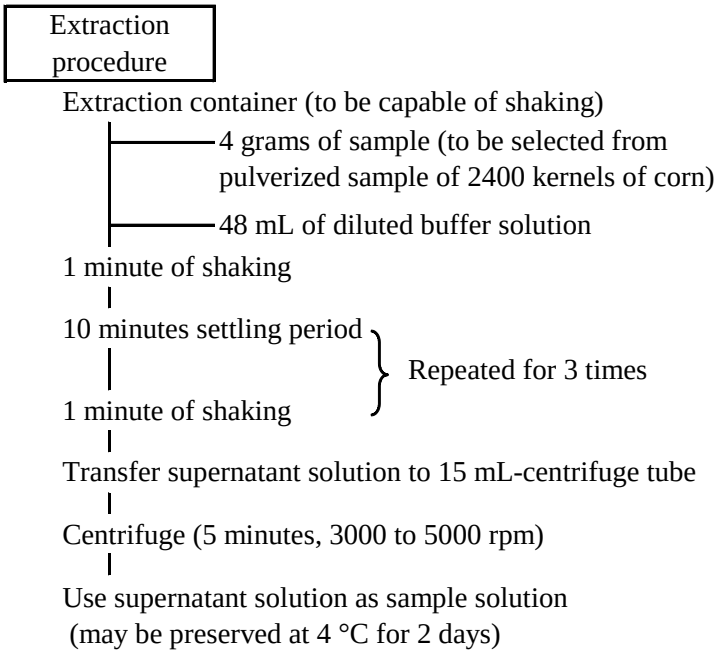
2.1.2.2.4. Determination

Calculate the mean of absorbance for respective standard samples and respective test samples. Prepare a calibration curve by the values of the standard samples, calculate the contamination rate of the test sample with CBH351 corn.

«Summary of the analysis method»

This method is to determine Cry9C protein using the ELISA assay:

Flowsheet for the analysis is indicated in figure 17.2-1 below:



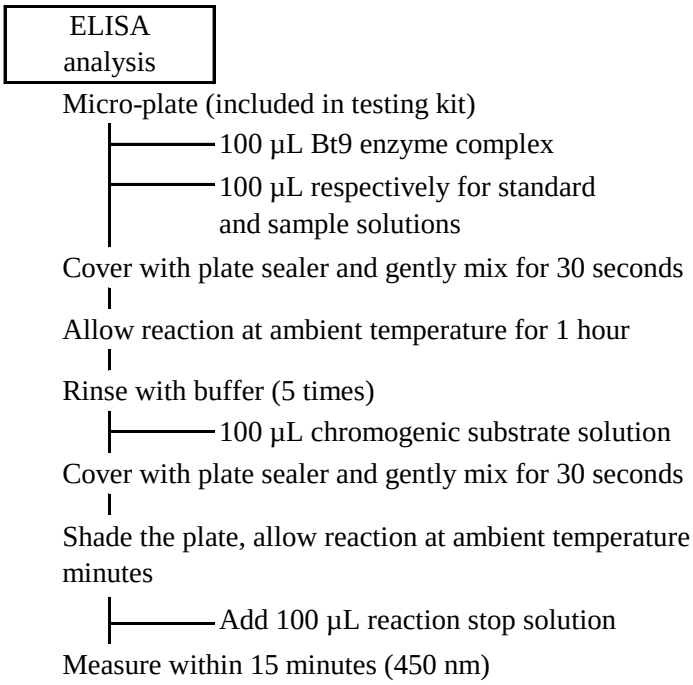


Figure 17.2-1 Flowsheet for determination of Cry9C protein by ELISA assay

«Method validation»

Limit of detection: Approximately 0.01 % as contamination rate.

«Notes and precautions»

[1] To reduce the difference in reaction time among wells, sequence for dropping reagents into wells shall be fixed, performing each procedure without unnecessary delay.

[A1] For points to be noted in performing the analysis procedure, refer to [Cautions for conducting inspections of feed derived from recombinant DNA technology].

[A2] Refer to «Notes and precautions» [A2] for subsection 2.1.1.

[A3] Precipitation may coagulate during the settling phase. Stir to lift precipitation prior to shaking.

[A4] To dispense reagents and samples, use variable micropipettes and (octal) multi-channel pipettes with capacity of about 10-100 μL or 100-1000 μL.

[A5] Required quantities of the samples shall be pre-arranged on alternate 96-hole plates, in the same sequence as the plate used in actual measurement, to be dispensed simultaneously using multi-channel pipette into the wells.

4 Analytical method for Bt10 corn [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.2.]

4.1 Qualitative PCR analysis of Bt10 corn [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.2.1.]

Inspection of corn kernels to be performed by a qualitative PCR method. Sample preparation shall be in accordance with the provisions of subsection 2.1.1.1. Excluding the contents of subsections 2.1.1.3.3.1. (Preparation of the PCR amplification product solution) and 2.1.1.3.5. (Judgment), the preparation of the PCR amplification product solution and the judgment for the qualitative PCR result shall comply with the description provided below in accordance with the provisions of subsection 2.1.1.3.[A1].

2.2.1.1. Preparation of the reaction solution for PCR

PCR amplification product solution shall be prepared for respective reaction tubes as indicated below. Add 5.0 μL of sample DNA solution pre-adjusted to 10 ng/μL concentration (equivalent to 50 ng of DNA) to a reaction tube immersed in water, containing a solution consisting of PCR buffer[1], 0.16 mmol/L dNTP, 1.5 mmol/L of magnesium chloride, 0.6 μmol/L respectively of 5′ and 3′ primers[2], and 0.8 units of DNA polymerase[3] to make overall quantity 25 μL. The reaction tube is then set onto the PCR thermal cycler[4]. Reaction conditions for the Bt10 identification primers are as indicated below[A2][A3]: Maintain 94 °C for 10 minutes to initiate the reaction, then allow the PCR amplification to proceed for 40 cycles with 94 °C for 25 seconds, 62 °C for 30 seconds and 72 °C for 45 seconds as one reaction cycle. Reaction conditions for the Bt10 confirmation primers are as indicated below: Maintain 94 °C for 10 minutes to initiate the reaction, then allow the PCR amplification to proceed for 40 cycles with 94 °C for 25 seconds, 65 °C for 30 seconds and 72 °C for 45 seconds as one reaction cycle[A4]. After 40 cycles, maintain 72 °C for 7 minutes and store sample at 4 °C. The end result will be used as the PCR amplification product solution. Concurrently to the preparation of the amplification product solution, a solution without primers and a solution not containing the DNA sample solution shall also be prepared for use as negative controls to the PCR reaction. Positive control plasmid[2] shall be provided as positive controls for the analysis. In order to confirm the extraction of DNA from the collected samples, PCR amplification using primers corresponding to the endogenous genes of corn (control primers) under the same reaction conditions as the Bt10 identification primers shall be performed for respective sample DNA solutions[A5].

2.2.1.2. Judgment

In the event the presence of 157 bp PCR amplification product corresponding to endogenous gene primers (control primers), as well as 117 bp PCR amplification product correponding to Bt10 detection primers[A6] and 151 bp PCR amplification product corresponding to Bt10 confirmation primers are all identified within either of the dually extracted samples as a result of the electrophoresis, the sample is deemed Bt10 positive.

When both extracted samples indicated the presence of the band of PCR amplification product of the control primers, but failed to indicate the band of PCR amplification product of the Bt10 detection

primers, the samples are deemed Bt10 negative. Even if the sample indicated the band of the Bt10 detection primers, it is deemed Bt10 negative in the event it failed to indicate the band of the Bt10 confirmation primers.

In the event either of the sample fails to indicate the presence of endogenous genes for the control primers, re-implement procedures from electrophoresis onward. If results do not change upon re-implementation, the DNA extraction sample shall be deemed invalid, and judgment shall be based on the results for the remaining sample. In case neither of the samples fail to indicate the presence of endogenous genes for the control primers, re-extract DNA (1 sample) and repeat the analysis. If the third DNA extraction sample also fails to indicate the presence of endogenous genes for the control primers, the detection of Bt10 from the subject sample shall be regarded as impossible. For examples of judgment, refer to the table provided in subsection 2.1.1.3.5.

«Summary of the analysis method»

The method is to detect Bt10 genes using PCR analysis:

Flowsheet for the analysis is indicated in figure 17.2-2 below:

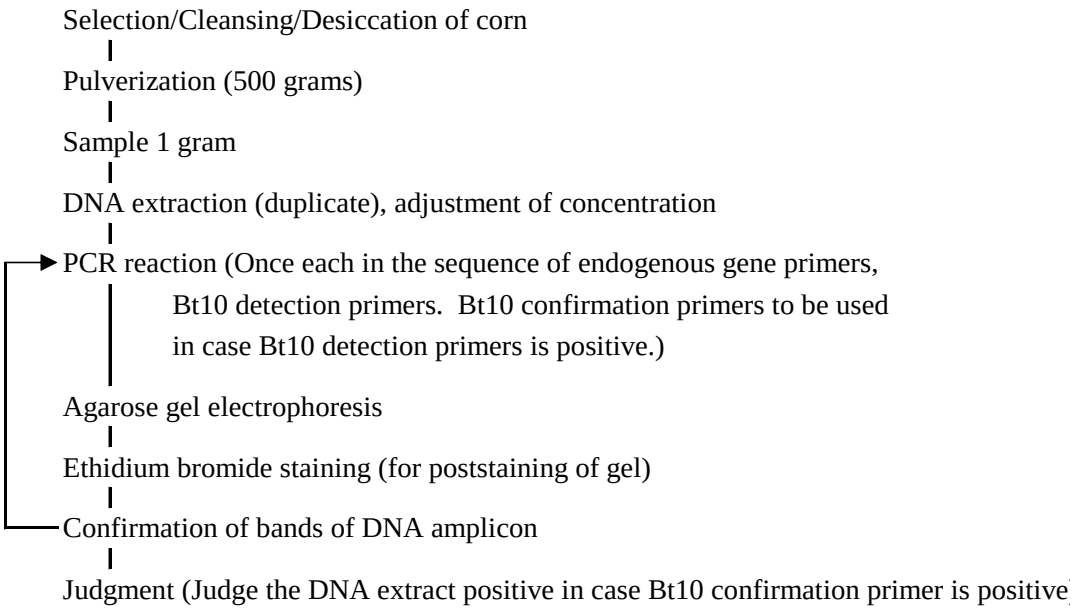


Figure 17.2-2 Flowsheet for detection of Bt10 genes by qualitative PCR

«Method validation»

Limit of detection: Approximately 0.05 % as contamination rate.

«Notes and precautions»

[1] PCR buffer: Use PCR buffer II (shall not contain magnesium chloride, manufactured by Applied Bio-Systems) or equivalent product.

[2] Primers and positive controls used in the analysis shall be as indicated below:

· GM Maize Detection Zein (for endogenous gene) Oligonucleotide: Nippon Gene (#318-05671) or FASMAC (#M9-1M)

· GM Maize Detection Bt10 (for 1st screening) Oligonucleotide: Nippon Gene (#317-06601) or FASMAC (#M11-1M)

Primer sequence to be as indicated below:

F-primer (JSF5): 5′-CAC ACA GGA GAT TAT TAT AGG GTT ACT CA-3′

R-primer (JSF5): 5′-ACA CGG AAA TGT TGA ATA CTC ATA CTC T-3′

· GM Maize Detection Bt10 (for 2nd screening) Oligonucleotide: Nippon Gene (#310-06601) or FASMAC (#M12-1M)

Primer sequence to be as indicated below:

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

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

· GM Maize Detection Bt10 Positive Control Plasmid: Nippon Gene (#317-06621) or FASMAC (#PM-5)

The reagents may be procured respectively from Nippon Gene Co., Ltd. (zip: 930-0983 1-8-7, Toiyamachi, Toyama-Shi; Tel. +81 76 451 6548, Fax. +81 76 451 6547) and FASMAC (zip: 243-0041 5-1-3 Midori-ga-Oka, Atsugi-Shi; Tel. +81 46 295 8787, Fax. +81 46 294 3738)

The text of the Notice included annotations regarding respective primers. Within this document, the contents of the annotations are rearranged as in the case of subsection 2.1.1.3.3.1. [6].

[3] DNA polymerase: Use AmpliTaq Gold® DNA polymerase (manufactured by Applied Biosystems) or equivalent product.

[4] PCR thermal cycler: Use GeneAmp® PCR System 9700 (manufactured by Applied Biosystems) or equivalent product..

[A1] Contrary to specifications for CBH351 analysis, the quantity of kernels to be used in the assay is defined as 500 grams. (Refer to **«Notes and precautions»** [1] for subsection 1 of this section: Sampling method).

[A2] Contrary to specifications for CBH351 analysis, remind that the conditions of PCR reaction for identification primers and confirmation primers are different.

[A3] To avoid misunderstandings, text “Bt10 detection primers” omitted in the original Notice was added.

[A4] Although the conditions of the reaction for the Bt10 detection primers was listed in the footnotes in the original Notice, but the conditions were inserted into the text portion for this document to indicate distinctively the difference of reaction conditions between the reaction using Bt10 confirmation primers and the reaction using Bt10 detection primers and endogenous gene primers.

[A5] Text “Bt10 detection primers” omitted in the original Notice was added.

[A6] The original Notice specifies the value 171 bp, which is an error. The correct value is 117 bp.

5 Analytical method for DAS59132 corn [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.3.]

5.1 Qualitative PCR analysis of DAS59132 corn [Method of Inspection for Feed Derived from Recombinant DNA Technology 2.3.1.]

Inspections of corn kernels to be performed by qualitative PCR method using a real-time PCR system[1]. Preparation of the samples shall be performed in accordance with the provisions of subsection 2.1.1.1[A1].

2.3.1.1. Primer sets and probes

2.3.1.1.1. Primer set and probe for corn positive control

Positive control for corn targets the starch synthase IIb (SSIIb) gene as the endogenous gene universally present in corn, and utilizes SSIIb-3[2] as a primer set and SSIIb-Taq[2] as a probe.

2.3.1.1.2. Primer set for detecting DAS59132[A2]

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

R-primer (32r): 5′-GGT GAA TGT CGC CGT GTG T-3′

Respective primers are water soluble.

2.3.1.1.3. Probe for detecting DAS59132[A2]

5′-FAM-CAA TTT GTT TAC ACC AGA GGC CGA CAC G-TAMRA-3′

Probe is water soluble.

2.3.1.2. Preparation of the reaction solution for PCR

The quantity of the reaction solution for PCR shall be 25 µL/well and shall consist of the following: 12.5 µL of universal PCR Master Mix[3], 1.0 µL of respective primer set solution (10 µmol/L of each primer) [4] and 0.5 µL of respective probe solution (10 µmol/L) shall be mixed, adjusted in quantity by adding water to achieve a total of 20 µL, then added with 5.0 µL (50 ng) of 10 ng/µL DNA sample solution[5]. Concurrently, prepare a solution without the DNA sample solution and adjusted with water to achieve an overall quantity of 25 µL as negative control for the PCR reaction[A3]. After completing the dispensing procedure, seal off well completely by applying seal perpendicularly[6]. To avoid corrugation, use dedicated sealing applicator for this procedure[7]. Observe bottom of the wells, and in the event bubbles are present at the bottom, lightly tap on rim of the plate to eliminate bubbles. After confirmation of the plate condition, set ABI PRISM Optical Cover Compression Pad[8] onto the top of the plate with the brown surface of the pad facing upward. Testing shall be conducted simultaneously in 2 wells per each DNA sample solution, requiring the preparation of the reaction solution for PCR in a quantity for 2 wells concurrently.

2.3.1.3. Setting of the plate information

Prior to initiating the reaction, the plate information, specifically the layout and type of sample and probe characteristics must be specified. Take a new sheet and set the type of sample (“UNKN”: DNA sample solution) with the entry corresponding to the layout of the prepared plate. Probe characteristics shall be set to corn positive control and DAS59132 detection, with the Reporter set to “FAM” and the Quencher to “TAMRA”. Along with the Passive Reference shall be set to “Rox” for both the corn

positive control and DAS59132 detection.

2.3.1.4. PCR

Set plate onto the real-time PCR system and initiate reaction, also starting data acquisition. Conditions of the reaction are as follows: Maintain 50 °C for 2 minutes, heat at 95 °C for 10 minutes, initiating reaction by hot start process. Amplification to be continued for 40 cycles with 95 °C for 15 seconds and 60 °C for 1 minute as one reaction cycle. Confirm that "Remaining time" has reached 0 minutes, then terminate reaction and analyze results.

2.3.1.5. Judgment

The results for corn positive control and DAS59132 detection are both judged based upon the exponential increase of amplification curve on the Amplification plot, the confirmation of Ct values and the distinctive exponential increase of fluorescence intensity (FAM) derived from the corresponding pigment on the multicomponent. Positive is assumed first by visual confirmation of the exponential increase in the curve on the Amplification plot. Then, select a Th. Line intersecting with the exponential amplification curve above the maximum noise range of ΔRn of the baseline (from 3 to 15 cycles). Analyze whether Ct values may be acquired from the selected Th Line. For both the corn positive control and DAS59132, judge results to be positive if Ct values less than 38 are obtained. In the event Ct values less than 38 cannot be obtained, the results are deemed negative. For results determined to be positive, analyze the multicomponent and visually confirm the exponential increase in the fluorescence intensity for FAM, and absence of a distinctive decline in the fluorescence intensity for ROX or gradual increase in the fluorescence intensity for FAM.

In the event either of the extraction sample fails to indicate Ct values below 38 for the corn positive control, re-implement procedures from real-time qualitative PCR onward. If results do not change upon re-implementation, the DNA extraction sample shall be deemed invalid, and judgment shall be based on the results for the remaining sample. In case neither of the samples fail to indicate Ct values below 38 for the corn positive control, re-extract and purify DNA (1 sample) and perform real-time qualitative PCR analysis. If the third DNA extraction sample also fails to indicate Ct values below 38 for the corn positive control, the detection of DAS59132 corn from the subject sample shall be regarded as impossible. For examples of judgment, refer to table provided in subsection 2.1.1.3.5. (However, the column for the confirmation primer is to be excluded.)

«Summary of the analysis method»

This method is to detectDAS59132 genes using real-time PCR analysis:

Flowsheet for the analysis is indicated in figure 17.2-3 below:

Selection/Cleansing/Desiccation of corn
↓
Pulverization (500 grams)
↓
Sample 1 gram
↓
DNA extraction (duplicate), adjustment of concentration
↓
Real-time PCR (Endogenous gene primer and probe, DAS59132 detection primer
↓ and probe to be reacted simultaneously on a single plate for the DNA solution)
Judgment (Positive in case both endogenous gene and DAS59132 are detected, negative if only endogenous gene is detected, other results require re-implementation of PCR)

Figure 17.2-3 Flowsheet for detection of DAS59132 genes by real-time PCR

«Method validation»

Limit of deteection: Approximately 0.05 % as contamination rate.

«Notes and precautions»

[1] Real time PCR: Utilize ABI PRISM™ 7700, ABI PRISM™ 7900HT, ABI PRISM™ 7500 or equivalent products.

[2] Primer set SSIIb-3 and probe SSIIb-Taq for corn positive control may be procured from Nippon Gene (#319-06061) or from FASMAC (M1-1M primer set and M1-2P probe).

[3] Universal PCR Master Mix (Applied Biosystems)

This reagent is viscous and shall be mixed with certainty to prevent failure in the PCR process. Mix for 3 seconds on the vortex mixer and lightly centrifuge for 3 seconds to collect reagent in the bottom of the tube prior to use. When dispensing in well, make sure the reagent is placed in the bottom of the well as stirring and centrifugation in subsequent processes are difficult to perform.

[4] Quantity of respective primer solution

When using primers (25 µmol/L) for corn positive control, add 0.5 µL of the solution.

[5] If possible, it is recommended to prepare a reaction solution based on positive control plasmid instead of the DNA sample solution.

[6] (ABI PRISM™ 7900, 7500) 96-well plate, seal and sealing applicator: Use MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems) and ABI PRISM Optical Adhesive Cover (Applied Biosystems). For details on the sealing, refer to a manual attached to the product.

[7] (ABI PRISM™ 7700) 96-well plate and plate cap: Use MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems) and MicroAmp Optical Caps, 8 caps/strips (Flat) (Applied Biosystems).

[8] If using ABI PRISM™ 7700, the procedure is not required.

[9] ABI PRISM Optical Cover Compression Pad: Use ABI PRISM Optical Cover Compression Pad (manufactured by Applied Biosystems). Avoid to reuse over 20 times may cause an adverse effect to quantitative outcome of the analysis. The pad is not applicable to ABI PRISM™ 7700 and ABI PRISM™ 7500.

[A1] Contrary to specifications for CBH351 analysis, the quantity of kernels to be used in the assay is defined as 500 grams. (Refer to **«Notes and precautions»** [1] for subsection 1 of this section: Sampling method). DNA extraction and purification from corn kernels shall be performed in

accordance with the provisions of subsection 2.1.1.3.1. and confirmation of the purity of undiluted DNA sample, preparation and storage of DNA sample solution shall be performed in accordance with the provisions of subsection 2.1.1.3.2. respectively.

[A2] As primer set and probe for detection of DAS59132 gene are not commercially available, these components must be prepared by DNA synthesis.

[A3] The text of the original Notice does not mention positive controls. However, positive controls shall be prepared concurrently with samples and negative controls in order to confirm the performance of PCR-related reagents, accuracy of analytical operations and the normal functions of the apparatus used. It is therefore recommended that tests using a positive control shall be implemented, at least for the easily obtained SSIIb-3. When implementing tests for the SSIIb-3 positive control, it is desirable to utilize GM corn positive control plasmid instead of DNA extraction solutions. Corresponding positive control plasmids are available from Nippon Gene (#314-04811) and FASMAC (PM-1). Currently positive controls corresponding to DAS59132 are not commercially available. DNA samples containing DAS59132 are also extremely rare, and preparation may prove to be difficult. But positive control plasmids of DAS59132 are desirable for use in testing the performance of the DAS59132 detection primers and probe. The use of plasmids is desirable in that steady Ct values are acquired and that contamination from positive control is easily identifiable by conducting PCR on the base structure of the plasmid in the event a cross-contamination between PCR reaction solutions is suspected.