Provisional inspection method of genetically modified flax (FP967)

The inspection target of this inspection method is flax grains. Extraction and purification of DNA is performed using an anion exchange resin-type kit method (Genomic-tip 20/G manufactured by QIAGEN) described below. Duplicate DNA extraction processes are performed from a sample; using the extracted sample DNA solutions, qualitative PCR using real-time PCR is performed. Flax grains are sampled in a manner similar to that described in "Enforcement of the Ministerial Ordinance, etc. to Partially Revise the Ordinance of the Ministry Concerning Standards and Specifications of Feeds and Feed Additives" (14SeiChiku No. 8598 as of April 1, 2003, latest revision 20Shoan No. 2496), Annex 3 Inspection method of feed derived from recombinant DNA technology, 1.1.1. Sampling of corn grains.

It should be noted that the safety of FP967 has been already confirmed in the United States, and therefore contamination limit of 1 % (Notice No. 1781 of the Ministry of Agriculture, Forestry and Fisheries of Japan, as of November 26, 2002) is applied to it. Accordingly, when contamination of FP967 is confirmed by the qualitative method using real-time PCR, the provisional determination (relative determination) that involves comparison with the Ct value ratio of a 1 % positive control solution shall be performed.

1. Extraction and purification of DNA from flax grains

A 0.5 g portion of a pulverized sample is weighed into a centrifuge tube (capacity: 50 mL) made of polypropylene, and DNA is extracted and purified as described below using an ion exchange resin-type DNA extraction purification kit (QIAGEN Genomic tip).

Then, 7.5 mL of G2 buffer solution[*1] and 20 μL of α-amylase[*2] are added to the sample. The mixture is vigorously mixed by a vortex mixer or like device, and the temperature of the mixture is kept at 37 °C for 1 h. Furthermore, 7.5 mL of G2 buffer, 200 μL of ProteinaseK[*3], and 20 μL of RNaseA[*4] are added to the mixture. The mixture is then stirred until the sample is no longer remaining at the bottom of the tube, and is maintained at 50 °C for 1 h. During that period, the centrifuge tube is inverted to mix the contents two to three times and then centrifuged at 5000×*g* and 4 °C for 15 min. The resulting supernatant is transferred into five 2-mL tubes in 2-mL portions (10 mL in total)[*5], and centrifuged at 20000×*g* and 4 °C for 15 min. A supernatant is collected in 1-mL portions from each of the 2-mL tubes and loaded onto QIAGEN Genomic-tip 20/G equilibrated with 1 mL of QBT buffer solution in advance[*5] (5 mL in total). Subsequently, the tips are washed with 2 mL of QC buffer[*1] three times, and then transferred to new centrifuge tubes. Then, 500 μL of QF buffer[*1] heated to 50 °C in advance is loaded onto the tip to elute DNA (eluate 1). The tips are transferred to new centrifuge tubes, and further DNA is eluted with 500 μL of QF buffer[*1] (eluate 2).

Subsequently, an equivalent volume of isopropanol is added to eluate 1 and eluate 2, respectively. The mixtures are slowly mixed by inversion 10 times, and are left to stand for 5 min at room temperature. The tubes are centrifuged at 12000×*g* and 4 °C for 15 min. The supernatant is discarded, and 500 μL of 70 % ethanol is added to each tube. The mixtures in

the tubes are mixed by inversion 10 times. The tubes are centrifuged at 12000×*g* and 4 °C for 3 min. The supernatant is discarded, and the remaining precipitates are dried to an appropriate degree. Then, 50 μL of water heated to 60 °C in advance is added to the centrifuge tube of eluate 2 to dissolve the precipitates. The entire solution is transferred to the centrifuge tube of eluate 1. The mixture is mixed well[*6], giving an extracted sample DNA solution. The DNA concentration of the extracted sample DNA solution is determined using a spectrophotometer.

*1 G2 buffer, QBT buffer, QC buffer, and QF buffer are enclosed in the kit, but can also be prepared according to the instruction in the kit for additional buffers.

*2 *α*-Amylase (high concentration) used is that manufactured by Nippon Gene, or that having a similar activity.

*3 Proteinase K used is that manufactured by Qiagen (20 mg/mL) or equivalents.

*4 RNaseA used is that manufactured by Qiagen (100 mg/mL) or equivalents.

*5 Care should be taken not to collect precipitates or upper layer film portions.

*6 When precipitates (DNA) are not dissolved, the tube is shaken to dissolve the precipitates at 65 °C for 15 min. When precipitates are not completely dissolved even then and insoluble substances are observed, the tube is centrifuged at 12000×*g* and 4 °C for 3 min and the resulting supernatant is transferred to a new centrifuge tube, which is used as an extracted sample DNA solution.

2. Qualitative PCR using real-time PCR

FP967 identification is performed by two real-time PCR tests using a primer pair and a probe for GM flax identification, and using a primer pair and probe for flax positive control.

A primer pair and a probe that detect a boundary region between NOS terminator and spectinomycin-resistant gene are used for GM flax identification. Moreover, a primer pair and a probe that detect stearoyl-acryl carrier protein desaturase 2 (SAD) gene sequence are used for flax positive control. These primers are soluble in water. The base sequences of the primers and probes are as follows:

Primer pair and probe for detecting GM flax

NOST-Spec F: 5′- AGC GCG CAA ACT AGG ATA AA-3′

NOST-Spec R: 5′- ACC TTC CGG CTC GAT GTC TA-3′

NOST-Spec probe: 5′-FAM- CGC GCG CGG TGT CAT CTA TG-BHQ1-3′

Primer pair and probe for flax negative control

SAD F: 5′- GCT CAA CCC AGT CAC CAC CT -3′

SAD R: 5′- TGC GAG GAG ATC TGG AGG AG -3′

SAD probe: 5′-FAM- TGT TGA GGG AGC GTG TTG AAG GGA-BHQ1-3′

2.1 Qualitative PCR and provisional determination (relative determination) using real-time PCR (ABI PRISM™ 7900)

2.1.1 Preparation of reaction solution for PCR

The reaction solution for PCR is prepared in an amount of 25 µL/well. The composition is as follows: 12.5 µL of Universal PCR Master Mix[*1], 0.4 µL each of respective primer pair solution (50 µmol/L for each primer), and 0.25 µL of respective probe solution (10 µmol/L) are mixed. Water is added to the mixture to make 22.5 µL of the solution in total, and 2.5 µL (125 ng) of a 50-ng/µL sample DNA solution is added to the mixture. Prepare 1 % positive control solution[*2] containing DNA derived from FP967 at a concentration of 1 %, use it as an FP967 positive control of PCR, and perform PCR together with the samples. Moreover, make sure to prepare a blank reaction mixture of PCR that does not contain the sample DNA solution.[*3] After the dispensing operation is completed, the wells are tightly sealed right from above.[*4] At this time, sealing is conducted using a special sealing applicator so that wrinkles are not formed. Finally, the bottoms of the wells are observed to check if there are air bubbles at the bottoms. If any, lightly tap the edge of the plate to remove the bubbles. After checking the plate, ABI PRISM Optical Cover Compression Pad[*5] is set on the upper face of the plate with its brown side facing up. Real-time PCR for both GM flax identification and flax positive control per each sample DNA solution in 2 wells each in parallel.

*1 Universal PCR Master Mix

Since this reagent has high viscosity, make sure mixing is thoroughly carried out in performing mixing operation. When mixing is insufficient, PCR may not be performed successfully. Immediately before use, make sure to mix the mixture for about 3 s using a vortex mixer, and centrifuge the mixture lightly to gather the solution at the bottom of the sample tube before use. Moreover, when dispensing into wells, considering that stirring and centrifuging will be difficult at further steps, make sure that the mixture is placed at the bottom of the wells.

*2 1 % Positive control solution

A 50-ng/µL DNA solution which is prepared by adding a pulverized FP967 flax seeds to a pulverized non-genetically modified flax seeds at a concentration of 1 % on a weight basis, and subjecting the mixture to extraction and purification of DNA, or equivalents.

*3 Non-Template Control (NTC)

When the sample DNA solution is dispensed, 2.5 µL of water is added to the wells of NTC instead of the sample DNA solution.

*4 96-well plate, seal, and sealing applicator

MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems), and ABI PRISM Optical Adhesive Cover (Applied Biosystems) are used. Refer to the manual enclosed with the product for the details of sealing.

*5 ABI PRISM Optical Cover Compression Pad

ABI PRISM Optical Cover Compression Pad (Applied Biosystems) is used. Do not use it more than 20 times since the measurement results may be affected.

2.1.2. Setting of plate information

Prior to the reaction, the plate information needs to be set. The items to be set are the arrangement and type of sample solutions, and probe characteristics. Specifically, setting of the type of the samples ("NTC": Non-Template Control, "UNKN": Sample DNA solution) is carried out on a new sheet while carefully checking correspondence to the arrangement of the plate prepared. For the probe characteristics, setting is made so that the Reporter of NOST-Spec and SAD is "FAM," while the Quencher is "Non Fluorescent." Moreover, the Passive Reference is set to "ROX." The selection of the run mode is set to the 9600 emulation mode.

2.1.3 PCR amplification

The plate is set in the device, and the reaction and data acquisition are started. The reaction conditions are as follows: Maintain 50 °C for 2 min, heat at 95 °C for 10 min, and a reaction is initiated by the hot start process. Thereafter, 45 cycles of amplification reaction are performed, with a cycle of 95 °C for 15 s and 60 °C for 1 min.

If it is confirmed that the remaining time is 0 min, the reaction is terminated, and analysis of the measurement results is performed.

2.2. Qualitative PCR and Provisional Identification (Relative Assay) Using Real-Time PCR (ABI PRISM 7700)

2.2.1 Preparation of reaction solution for PCR

The reaction solution for PCR is prepared in an amount of 25 µL/well. The composition of the solution is as follows: 12.5 µL of Universal PCR Master Mix[*1], 0.4 µL each of respective primer pair solutions (50 µmol/L of each primer), and 0.25 µL of respective probe solution (10 µmol/L) are mixed. Water is added to the mixture to make 22.5 µL in total, and 2.5 µL (125 ng) of a 50-ng/µL sample DNA solution is added to the mixture. Prepare 1 % positive control solution[*2] containing DNA derived from FP967 at a concentration of 1 %, use it as an FP967 positive control of PCR, and perform PCR together with the samples. Moreover, a solution with no sample DNA solution added is always prepared simultaneously as a blank reaction solution of PCR.[*3] After the dispensing operation is completed, the wells are tightly sealed right from above.[*4] At this time, sealing is conducted using a special sealing applicator so that wrinkles are not formed. Finally, the bottoms of the wells are observed to check if there are air bubbles at the bottoms. If any, lightly tap the edge of the plate to remove the bubbles. Real-time PCR for both GM flax identification and flax positive control per each sample DNA solution in 2 wells each in parallel.

*1 Universal PCR Master Mix

Since this reagent has high viscosity, make sure mixing is thoroughly carried out in

performing mixing operation. When mixing is insufficient, PCR may not be performed successfully. Immediately before use, make sure to mix the mixture for about 3 s using a vortex mixer, and centrifuge the mixture lightly to gather the solution at the bottom of the sample tube before use. When dispensing into wells, considering that stirring and centrifuging will be difficult at further steps, make sure that the mixture is placed at the bottom of the wells.

*2 1 % Positive control solution

A 50-ng/μL DNA solution which is prepared by adding a pulverized FP967 flax seeds to a pulverized non-genetically modified flax seeds at a concentration of 1 % on a weight basis, and subjecting the mixture to extraction and purification of DNA, or equivalents.

*3 Non-Template Control (NTC)

When the sample DNA solution is dispensed, 2.5 μL of water is added to the wells of NTC instead of the sample DNA solution.

*4 Well plate, seal, and sealing applicator

MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems), and ABI PRISM Optical Adhesive Cover (Applied Biosystems) are used. Refer to the manual enclosed with the product for the details of sealing.

2.2.2. Setting of plate information

Prior to the reaction, the plate information needs to be set. The items to be set are the arrangement and type of sample solutions, and probe characteristics. Specifically, setting of the type of the specimens (“NTC”: Non-Template Control, “UNKN”: Sample DNA solution) while carefully checking correspondence to the arrangement of the plate prepared on a new sheet is carried out. For the probe characteristics, setting is made so that the Reporter of NOST-Spec and SAD is “FAM,” while the Quencher is “Non Fluorescent.” Moreover, the Passive Reference is set to “ROX.” The selection of the run mode is set to the 9600 emulation mode.

2.2.3 PCR amplification

The plate is set in the device, and the reaction and data acquisition are started. The reaction conditions are as follows: Maintain 50 °C for 2 min, heat at 95 °C for 10 min. A reaction is initiated by the hot start method. Thereafter, 45 cycles of amplification reaction are performed, with each cycle of 95 °C for 15 s and 60 °C for 1 min. If it is confirmed that the remaining time is 0 min, the reaction is terminated, and analysis of the measurement results is performed.

3. Judgment and analysis of the results

3.1 Qualitative method

For both the test for GM flax identification and the test for flax positive control, the judgment of the results is performed by the confirmation of an exponential amplification curve and a Ct value on the amplification plot, and the confirmation of a distinctive exponential increase in the fluorescence intensity derived from the target pigment (FAM) on

the multicomponent.

First, when the exponential amplification curve of NOST-Spec is visually confirmed on the amplification plot, FP967 positive is suspected. Subsequently, a threshold line (Th. line) that crosses the stable exponential amplification curve is chosen above the maximum value of the noise band of ΔRn of the baseline (3 cycles to 15 cycles). Analyze whether the Ct value is obtained from the Th. line. When there is a well from which a Ct value lower than 43 is obtained in the test for flax positive control and a Ct value lower than 43 is obtained in the test for the GM flax identification in the sample DNA solutions, it is judged FP967 positive. When a Ct value lower than 43 is obtained in the test for flax positive control and a Ct value lower than 43 is not obtained in the test for GM flax identification, it is judged FP967 negative. When the results in the two sample DNA solutions are different, it is judged positive. The multicomponent is analyzed for the results of FP967 positive by the above-mentioned judgment to check that an exponential increase in the fluorescence intensity of FAM can be observed visually, and that there is no clear drop in the fluorescence intensity of ROX or a moderate rise in the fluorescence intensity of FAM.

3.2 Provisional determination method

For the sample judged as FP967 positive in 3.1, obtain Ct values of the test for GM flax identification and the test for flax positive control, and calculate the ratio of Ct value of the test for GM flax identification / Ct value of the test for flax positive control (Ct value ratio). Likewise, a Ct value ratio of the 1 % positive control solution performed on the same plate with the sample. When the Ct value ratio of the sample is lower than the Ct value ratio of the 1 % positive control solution, it is determined that FP967 is contained in an amount higher than 1 % in the sample.

3.3 Retest

About the sample DNA solution in which less than 43 Ct value is not obtained for flax positive control, perform the qualitative PCR method using real-time PCR again. If a Ct value lower than 43 is not obtained, the result of the sample DNA solution is determined invalid, and judgment for the sample is made only by the result of the sample DNA solution that provided a Ct value lower than 43. When a Ct value lower than 43 is not obtained in the test for flax positive control with both sample DNA solutions, perform the third extraction and purification of DNA and the qualitative PCR using real-time PCR. When a Ct value lower than 43 is not obtained in the test for flax positive control even when the third sample DNA solution is used, it is determined that detection from this sample is impossible.

* It is difficult to show a numerical value of the setting of a general Th. line due to a variation in ΔRn on the amplification plot depending on the status of each model. Therefore, a Th. line that crosses a stable exponential amplification curve further above the maximum value of the noise band of ΔRn of the baseline (3 cycles from 15 cycles) on the amplification plot is selected. For information, it is supposed that ABI PRISM™ 7900 and ABI PRISM™ 7500 both have Th. values ranging from 0.2 to 0.5.