Provisional inspection method of genetically modified wheat (MON71800)

1. Scope of inspection

The target of this assay is the wheat grain.

When the contamination of MON71800 is confirmed by real-time PCR method in 4, perform the determination tests for 1 % contamination in 5.2.

2. Sampling method of wheat grain

Sampling of wheat grain should be performed in accordance with 'the method of inspection for feed derived from recombinant DNA technology 1. Sampling method' (attachment 3 of ministerial notice No. 8598 dated April 1st, 2003 by director general of Production Bureau, Ministry of Agriculture, Forestry and Fisheries). The total amount of grain taken are washed and crushed in 3.1.

3. Extraction and purification of DNA from wheat grains

3.1. Washing and crushing of the sample

Stir wheat grains in 1 % SDS aqueous solution of three volumes of sample (by weight). Use a container of five volumes of sample. After that, rinse until no bubbles out with ion-exchanged water or distilled water. Spread washed grains on top of a paper towel and let them dry for 40 minutes in a dryer of 40 °C. Grind grains to homogeneity using a grinder or the like, to prepare the storage sample and the test sample.

3.2. Method using silica gel membrane type kit (DNeasy Plant Maxi Kit, QIAGEN)

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

Measure and transfer 1.0 g of the sample into a 50-mL centrifuge tube, add 10 µL of 100 mg/mL RNaseA[*2] and 5 mL of API buffer preheated to 65 °C 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 two or three times. Then, add 1.8 mL of Buffer P3[*4] (former name: AP2 buffer) to the tube, agitate with the vortex tube mixer and cool in ice-cold water for 15 minutes. Centrifuge the sample for 15 minutes in room temperature at 3000×*g* using a swing centrifuge. Collect 4.5 mL of supernatant solution, load onto the QIAshredder Maxi spin column and centrifuge for 5 minutes in room temperature at 3000×*g*. Transfer 4 mL of supernatant solution to a new 50-mL centrifuge tube. Add 6 mL of Buffer AW1[*5] (former name: Buffer AP3/E) and agitate vigorously on the vortex tube mixer. Load the entire solution onto the DNeasy Maxi spin column and centrifuge for 5 minutes in room temperature at 3000×*g*. Discard the eluate and load 12 mL of Buffer AW2[*6] (former name: Buffer AW) onto the column. Centrifuge for 15 minutes in room temperature at 3000×*g*. Transfer the column to a new 50-mL tube, add 1 mL of sterilized ultra-pure 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*. Transfer the eluate to a 2 mL-tube. Add isopropanol equivalent in amount to the eluate. 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 70 % ethanol and

wash away precipitate by tapping the bottom of the tube. Centrifuge the tube for 3 minutes at 4 °C, 12000×*g*. Discard supernatant solution thoroughly[*7]. Allow precipitate to dry, add 100 µL of sterilized ultra-pure water to dissolve precipitate. After confirmation of the absence of insoluble substance[*8], the resulting solution shall be used as the undiluted sample DNA solution.

*1 Throughout the experiment, be careful enough so that contamination between samples does not occur, like to exchange and chips pipette for dispensing and chips for each sample.

*2 Those included in the kit, those purchased from QIAGEN (Cat. no. 19101) separately or equivalents

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*5 Those included in the kit or those purchased from QIAGEN (Cat. No. 19081) separately

*6 Those included in the kit or those purchased from QIAGEN (Cat. No. 19072) separately

*7 Even if the precipitate is not visible, remove the supernatant so as not to touch as much as possible near the bottom of the centrifuge tube. The operation is repeated to tap the tube with fingers, centrifuge and remove the liquid droplets from the wall.

*8 If the insoluble matter is observed, allowed to stand for 12-24 hours in a refrigerator. Any insoluble matter is observed even after 24 hours, centrifuge for 3 min at 12000×*g* and 4 °C, transfer the supernatant to a new tube to be the DNA sample stock solution. In addition, store the precipitate at −20 °C.

3.3. Confirmation of the purity, preparation and storage of sample DNA

Take an appropriate amount of the undiluted DNA sample and dilute it[*1] by adding sterilized ultra-pure water for analysis using the spectrophotometer. Measure the UV absorption in the range of 200 to 320 nm to acquire the absorbance in 260 nm, and 280 nm (A260 and A280)[*2]. Calculate the DNA concentration upon an assumption that the DNA concentration when A260 = 1 is 50 ng/µL. If the ratio of A260 / A280 is within the range of 1.7 to 2.0, the DNA is sufficiently purified[*3]. Based on the calculated DNA concentration of the undiluted sample DNA solution, prepare a 10 ng/µL sample DNA solution, adjusting the concentration by diluting with sterilized ultra-pure water. Dispense solution into 0.5-mL or 1.5-mL micro-tube by 40 µL per tube to be cryopreserved at below −20 °C. Dispensed DNA solutions shall be used in the analysis immediately upon thawing, and discard the residue. In the case that the concentration of undiluted sample DNA solution is lower than 10 ng/µL, the solution shall be used without adjustment.

*1 Set dilution factor, according to appropriate volume and concentration of solution for each spectrophotometer.

*2 A260 is considered as the absorbance of DNA. A280 is considered as the absorbance of impurities such as protein.

*3 Even if the ratio of A260 / A280 is outside the range of 1.7-2.0, it does not require the further operation such as purification.

4. Qualitative real-time PCR method (ABI PRISM™ 7900*)

Perform qualitative real-time PCR for each sample DNA solution prepared in 3 using two wells per sample.

Detection of genetically modified wheat (MON71800) is determined by 2 tests of real-time PCR using primers and probe for wheat positive control test, and for MON71800 identification test. For MON71800 identification test, primers and probe to detect the boundary region between vector for cp4epsps gene expression and wheat genome sequence are used. Further, for the wheat positive control test, primers and probe for detecting proline-rich protein (PRP) gene sequence are used. Each primers and probe are dissolved in sterilized ultra-pure water. Nucleotide sequences of primers and probes are as follows.

* It is possible to use other models having a performance equivalent to ABI PRISM™ 7900.

Primer pair and probe for identification of MON71800

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

SQ0719: 5′-CCC CCA TTT GGA CGT GAA-3′

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

Primer pair and probe for positive control of wheat

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

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

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

4.1. 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 FastStart Universal Probe Master (Rox) (Roche Diagnostics)[*1], 0.25 µL of respective primer pair solution (50 µmol/L of each primer) 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 µL of 10 ng/µL sample DNA sample solution[*2].

Concurrently, prepare a solution without sample DNA solution and adjusted with water to achieve an overall quantity of 25 µL as blank for the PCR reaction[*3].

After completing the dispensing procedure, seal off well completely by applying seal perpendicularly[*4]. To avoid corrugation, use dedicated sealing applicator. 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 MicroAmp

Optical Cover Compression Pad (Life Technologies)[*5] 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 sample DNA solution, for both MON71800 identification test and positive control wheat test.

*1 It is possible to use TaqMan® Universal PCR Master Mix (Life Technologies) or Eagle Taq Master Mix (Rox) (Roche Diagnostics) instead of FastStart Universal Probe Master (Rox) (Roche Diagnostics).
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.

*2 Needed amount of frozen reagents picked out from the freezer, should be thawed at room temperature, and stored on ice. For each reagent placed on ice, if they are dispensed continuously through using the same chip, the air in the pipette is cooled, and the second and subsequent portions would not be dispensed exactly. Used to understand the procedure when working with low-temperature sample (typically, operation called Fukitome) written in instructions of pipette.

*3 For Non-Template Control (NTC), add 5μL of sterilized ultra-pure water to the wall instead of sample DNA solution.

*4 MicroAmp Optical 96-Well Reaction Plate (Life Technologies) is used as 96-well plate. ABI PRISM Optical Adhesive Cover (Life Technologies) is used as seal. Refer to attached manual for details in sealing.

*5 Use for ABI PRISM™ 7900, but not for ABI PRISM™ 7500.

4.2. 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 (“NTC”: Non-Template Control, “UNKN”: DNA sample solution) with the entry corresponding to the layout of the prepared plate. Probe characteristics shall be set for MON71800 identification test with the Reporter set to “FAM” and the Quencher to “Non Fluorescent” and for wheat positive control test with the Reporter set to “FAM” and the Quencher to “TAMRA”. Along with the Passive Reference shall be set to “ROX”. Run mode shall be set to 9600 emulation mode. Sample volume shall be set to 25 μL.

4.3. PCR amplification

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 45 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.

5. Judgment and analysis of the results

5.1. Qualtitative real-time PCR method

The results for MON71800 identification test and wheat positive control test 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 derived from the corresponding pigment (FAM) on the multicomponent. MON71800 positive is assumed first by visual confirmation of the exponential increase in the curve on the Amplification plot. Then, set Th. Line to 0.2 as far as it is above the maximum noise range of ΔRn of the baseline set from 3 to 15 cycles, and it is intersecting with the stable exponential amplification curve. If the Th. Line intersects either with noise or non-exponential amlplification curve, modify the Th. Line not to intersect both of them. Analyze whether Ct values may be acquired from the selected Th Line. At first, judge the results of duplicate extracted sample DNA solutions (each 2 wells) following the scheme below.

For each sample DNA solution,

(1) Judge the results to be positive if Ct values for MON71800 identification test less than 43 are obtained in both 2 wells, as well as Ct values for wheat positive control test less than 43 are obtained in both 2 wells.

(2) Judge the results to be negative if Ct values for MON71800 identification test less than 43 are not obtained in either 2 wells, while Ct values for wheat positive control test less than 43 are obtained in both 2 wells.

(3) Re-implement the procedures from “1. Extraction and purification of DNA” onward if Ct values for MON71800 identification test in 2 wells are inconsistent, while Ct values for wheat positive control test less than 43 are obtained in both 2 wells.

The sample shall be judged to be positive if both of duplicate extracted sample DNA solution (4 wells in total) is positive, and to be negative if at least 1 sample DNA solution is negative. In case of (3), judge as negative if the result of re-implemented sample DNA solution is not positive.

For results determined to be MON71800 positive, analyze the multicomponent. Confirm that the exponential increase in the fluorescence intensity for FAM or VIC is observed visually, and a distinctive decline in the fluorescence intensity for ROX and gradual increase in the fluorescence intensity for FAM or VIC are absent.

In case neither of the samples fail to indicate Ct values below 43 for the wheat positive control test at least 1 well, re-extract and purify DNA and perform real-time qualitative PCR analysis. If the sample DNA solution also fails to indicate Ct values below 43 for the wheat positive control test, the detection of MON81700 from the sample shall be regarded as impossible.

5.2 Determination test for 1 % contamination

For samples judged to be MON71800 positive (Hereinafter referred to as "positive samples")

in 5.1, perform a determination test to confirm if the contamination ratio of MON71800 is greater than 1 %.

In this test, perform real-time PCR using positive samples and 1 % positive control solution[*1] simultaneously. For operation procedure, refer to "4. Qualitative real-time PCR method".

Prior to judgement, calculate the difference of Ct values (ΔCt) between MON71800 identification test and wheat positive control test for both of positive samples and 1 % positive control solution. Further, calculate the difference of ΔCt (ΔΔCt) between positive samples and 1 % positive control solution.

(1) If ΔΔCt value is positive: Since the PCR amplification amount of positive samples requires a number of cycles more than the 1 % positive control solution to reach a threshold value, contamination of MON71800 in the positive samples is not more than 1 %.

(2) If ΔΔCt value is negative: Since the PCR amplification amount of positive samples does not require a number of cycles than the 1 % positive control solution to reach a threshold value, contamination of MON71800 in the positive samples is greater than 1 %.

*1 1 % positive control solution is prepared by following methods I, II or equivalents.

I Sample DNA (provided by Monsanto) is prepared by mixing one grain of MON71800 and 14 grains of non-genetically modified wheat and crushing. Purify the sample DNA using DNeasy Plant Mini Kit (QIAGEN) or equivalents, adjust the DNA concentration to 10 ng/μL with sterilized ultrapure water. Then adjust the concentration of MON71800 to 1 % with sample DNA solution (which is derived from non-genetically modified wheat grain and adjusted the DNA concentration to 10 ng/μL as prescribed in "3. Extraction and purification of DNA from wheat grains").

II Mix crushed MON71800 grains into crushed non-genetically modified wheat grains as to be 1 % on a weight basis. Then, using this mixture, prepare sample DNA solution of 10 ng/μL in DNA concentration as prescribed in "3. Extraction and purification of DNA from wheat grains").