Validation Study

Interlaboratory Validation of Quantitative Duplex Real-Time PCR Method for Screening Analysis of Genetically Modified Maize

(Received March 4, 2011)

Reona TAKABATAKE[1], Tomohiro KOIWA[2], Masaki KASAHARA[2], Kaori TAKASHIMA[1], Satoshi FUTO[3], Yasutaka MINEGISHI[4], Hiroshi AKIYAMA[5], Reiko TESHIMA[5], Taichi OGUCHI[1], Junichi MANO[1], Satoshi FURUI[1] and Kazumi KITTA[1,*]

[1]Analytical Science Division, National Food Research Institute, National Agriculture and Food Research Organization: 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan; [2]Food and Agricultural Materials Inspection Center: 2-1 Shintoshin, Chuo-ku, Saitama, Saitama 330-9731, Japan; [3]FASMAC: 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Japan; [4]NIPPON GENE: 1-8-7 Toiyamachi, Toyama, Toyama 930-0834, Japan; [5]National Institute of Health Sciences: 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; * Correspoding author

To reduce the cost and time required to routinely perform the genetically modified organism (GMO) test, we developed a duplex quantitative real-time PCR method for a screening analysis simultaneously targeting an event-specific segment for GA21 and Cauliflower Mosaic Virus 35S promoter (P35S) segment [Oguchi *et al.*, J. Food Hyg. Soc. Japan, 50, 117-125 (2009)]. To confirm the validity of the method, an interlaboratory collaborative study was conducted. In the collaborative study, conversion factors (Cfs), which are required to calculate the GMO amount (%), were first determined for two real-time PCR instruments, the ABI PRISM 7900HT and the ABI PRISM 7500. A blind test was then conducted. The limit of quantitation for both GA21 and P35 S was estimated to be 0.5% or less. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation (RSD_R). The determined bias and RSD_R were each less than 25%. We believe the developed method would be useful for the practical screening analysis of GM maize.

Key words: screening; quantification; genetically modified (GM); duplex real-time PCR; maize (*Zea mays*)

Introduction

The PCR technique is widely used to detect and quantify GM crops in foods and feeds. The key factor determining the specificity of a PCR-based method is the choice of a target sequence motif in the GM plant genome. The methods can be classified into at least 3 categories depending on the target; event-specific, construct-specific and screening methods. In event-specific methods, a unique sequence located at the junction between the plant genome and recombinant DNA is used as the target. Construct-specific methods target the junction between adjacent elements in an introduced gene cassette, such as a region between a promoter and a structural gene. Screening methods target commonly conserved elements among many GM events such as Cauliflower Mosaic Virus 35S promoter (P35S), nopaline synthase terminator, 5-enolpyruvylshikimate-3-phosphate synthase, phosphinothricin *N*-acetyltransferase, and so on[1)-4)]. Event-specific methods are the most specific, followed by construct-specific and screening methods. Screening methods often overestimate GM amounts, and it is generally agreed that the best estimates can be obtained by event- or construct-specific quantifications. In fact, construct-specific quantitative methods of five GM maizes (Bt11, Bt176, GA21, MON810, T25) and Roundup Ready Soy (RRS) have been adopted as Japanese standard analytical methods[*1,*2]. However, the cost of genetically modified organism (GMO) testing using specific quantifications

* E-mail: kaz@affrc.go.jp

[*1] Notification No. 110 (Mar. 27, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001).

will increase in parallel with the number of GM events to be examined. In this regard, screening methods are highly cost- and time-effective for routine monitoring. The Ministry of Health, Labour and Welfare (MHLW) of Japan announced a screening method combining the quantification of a P35S region and the construct-specific quantification of GA21 maize, which has been officially used as a quantitative screening method for GM maize[*3]. To further pursue more convenient and efficient methodology, we developed a duplex real-time PCR method which simultaneously quantifies the P35S region and an event-specific segment of GA21[5]. The developed duplex screening method will reduce both the cost and time requirement of routine GMO analysis by half compared to the current screening method. These quantitative methods are based on a real-time PCR technique for relative quantification between target and taxon-specific sequences. In many cases, extracted DNAs from processed foods are severely degraded and the degree of the degradation is not always the same among PCR-targeted sequences, so that GM quantification in processed foods by means of the PCR technique is difficult[6),7]. In fact, the quantification methods adopted as Japanese standard analytical methods, including the current screening method, are applicable to raw materials but not to processed foods.

In this report, we validated the duplex real-time PCR method for the screening analysis of GM maize by means of an interlaboratory study.

Materials and Methods

Plant materials

The GM maize seeds, MON810 and GA21 were kindly provided by Monsanto (St. Louis, MO, USA), and Syngenta Seeds (Basel, Switzerland), respectively. MRX 3 maize was purchased from Pioneer Hi-Bred International (Johnston, IA, USA) and used as a non-GM maize sample.

Oligonucleotide primers and probes

All primers and probes used in this study were identical to those in the previous single laboratory evaluation[5]. The primers and probe for the event-specific detection of GA21 were as follows: GA21esp 5′-1,5′-TGGGACCTTATCGTTATGCTATTTG-3′; GA21esp3′-1,5′-CGATCCTCCTCGCGTTTCC-3′; and GA21es-TaqHB; 5′-CCGGACCCACCTGCTGTTGAGAAAG-3′. The primers and probe for the detection of the P35S region were as follows: P35S 1-5′,5′-ATTGATGTGATATCTCCACTGACGT-3′; P35S 1-3′,5′-CCTCTCCAAATGAAATGAACTTCCT-3′; and P35S-TaqFB, 5′-CCCACTATCCTTCGCAAGACCCTTCCT-3′. We used the maize *starch synthase IIb* (*SSIIb*)[9] gene as a maize-specific endogenous DNA for quantitative analysis, and the primers and probe for *SSIIb* were as follows: SSIIb3-5′,5′-CCAATCCTTTGACATCTGCTCC-3′; SSIIb3-3′,5′-GATCAGCTTTGGGTCCGGA-3′; and SSIIb-TaqFB, 5′-AGCAAAGTCAGAGCGCTGCAATGCA-3′. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC (Kanagawa, Japan) and Biosearch Technologies (Novato, CA, USA), respectively. All synthesized probes were labeled with Blackhole Quencher (BHQ) at the 3′ ends, and P35S-TaqFB and SSIIb-TaqFB were labeled with 6-carboxyfluorescein (FAM), while GA21es-TaqHB was labeled with hexachloro-6-carboxy-fluorescein (HEX), at the 5′ ends.

Preparation of calibrant plasmid

The standard plasmid pSCM which contains the specific sequence fragments from GA21, P35S and *SSIIb*, was prepared according to the previous report[5] and used as the calibrator for the quantification.

Preparation of test samples

Non-GM and GM maize mixing samples containing 0.50, 1.0, 5.0, and 10.0% of both GA21 and MON810 were prepared by mixing dried powders in the manner described previously[9),10] and used as a primary certified reference material. Briefly, washed maize seeds of non-GM, GA21 and MON810 were separately frozen, ground with a high-speed rotor mill (Fritsch, Idar-Oberstein, Germany), freeze-dried in a freeze dryer (FDU-1100; Tokyo Rikakikai, Tokyo, Japan), and then mixed on a weight-to-weight basis.

Quantitative PCR

All conditions and PCR instruments were identical to those in the previous report[5].

Homogeneity of test samples

Test samples of each GM mixing level were aliquoted (1 g each) into 200 sample tubes. Ten sample tubes were randomly selected twice from the 200 tubes prepared. DNA was extracted from each sample using the DNeasy Plant Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's manual, and quantitative PCR was performed using GA21 and MON810 construct-specific methods[8)–10]. The calculated copy numbers were then converted into GMO amounts (%) on a weight basis. The homogeneities of GA21 and MON810 were independently evaluated by one-way analysis of variance (ANOVA) as described previously[8)–10].

Interlaboratory study

The interlaboratory study consisted of 2 separate stages, a measurement of the Cf values and a blind test. Experimental protocols were provided by the Food and Agricultural Materials Inspection Center (FAMIC). The Universal Master Mix, primers, probes, blind samples

[*2] Japanese Agricultural Standard (JAS) analytical test handbook: genetically modified food quality, labeling analysis manual for individual products (2002). The Food and Agricultural Materials Inspection Center, Japan. http://www.famic.go.jp/technical_information/jashandbook/index.html

[*3] Notification No. 618001; Department of Food Safety, Ministry of Health, Labour and Welfare of Japan: Tokyo, Japan, 2003.

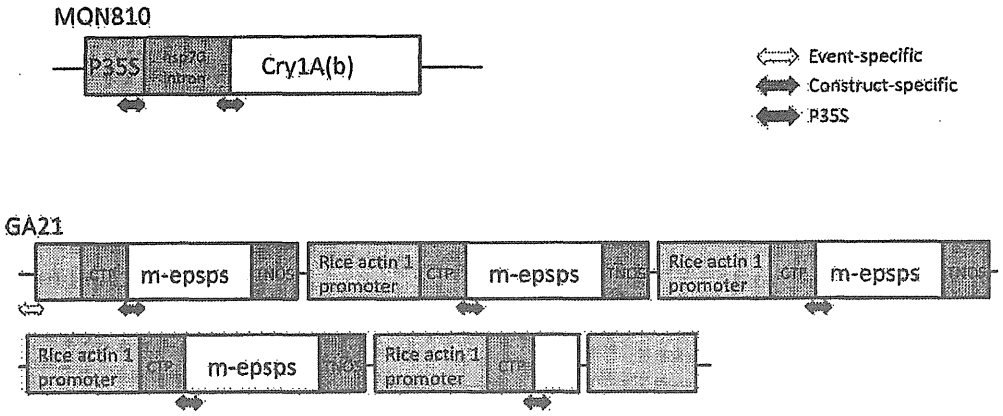

Fig. 1. Schematic diagrams showing the target positions in MON810 and GA21

The event-specific, construct-specific, and universal P35S target sequences are indicated with white, gray, and black double-headed arrows, respectively.

Table 1. Summary of the Cf values for ABI PRISM 7900 and 7500

	7900			7500		
	Mean	SD	RSD	Mean	SD	RSD
GA21	0.375 (0.38)[a)]	0.046	12.3	0.332 (0.33)	0.025	7.60
	Mean	SD	RSD	Mean	SD	RSD
P35S	0.364 (0.36)	0.024	6.50	0.363 (0.36)	0.013	3.60

SD: Standard deviation
RSD: Relative standard deviation
[a)] The mean values rounded to the nearest hundredth of a unit are shown in parentheses.

and DNeasy Plant Maxi kit were also supplied by FAMIC.

The first stage was measurement of the Cf values using the ABI 7900HT (AB 7900) and the ABI 7500 (AB 7500) (Life Technologies, Carlsbad, CA, USA). The Cf value is experimentally determined as the ratio of the copy number of r-DNA to the taxon-specific sequence in the GM plant genome. The Cf values for GA21 and P35 S were determined independently from the results of 12 laboratories for the AB 7900, and 5 laboratories for the AB 7500. The measurements were repeated 3 times in each laboratory, and the average values from all the submitted data were defined as the Cf values. After determination of the Cf values, one laboratory withdrew from this study.

The blind test was conducted as the second stage. All measurements were performed by 11 laboratories for the AB 7900. The maize samples were designed as blind duplicates, including 0, 0.5, 1.0, 5.0 and 10.0% of both GA21 and MON810. The blind samples sent to the participants were divided into two sets containing each concentration, and then the measurements were separately performed for each set of blind samples. DNAs were extracted from these blind samples by each participant and then quantitative analyses were carried out. All participants were requested to submit the data from the real-time PCR analyses. All submitted data were analyzed by means of Cochran's test[11] and Grubbs' test[12), 13)]as described in the guidelines[14].

Results and Discussion

Determination of Cf values for GA21 and P35S

The Cf value for GA21 was determined by measuring the copy numbers of endogenous gene *SSIIb* and GA21 in the extracted DNA from the GA21 seed. To determine the Cf value for P35S, we used MON810 as a representative of GM maize both because it has been widely used, and because it has only one P35S segment per GM haploid, as the previous single laboratory evaluation described[5]. The Cf values for GA21 and P 35S were measured independently with two real-time PCR instruments, the AB 7900 and AB 7500. The Cf values determined are listed in Table 1. The values for P35S with AB 7900 and AB 7500 were very close, and when rounded to the nearest hundredth of a unit both values became 0.36. GA21 contains a single insert

Table 2. Homogeneity of the simulated mixtures

	% (w/w)	Measured mean, %	F-value[a]	p-value
GA21	0.50	0.44	1.01	0.49
MON810	0.50	0.48	1.82	0.18
GA21	1.0	0.74	0.48	1.00
MON810	1.0	0.95	0.11	0.86
GA21	5.0	3.71	0.47	0.87
MON810	5.0	5.26	0.83	0.60
GA21	10.0	7.67	1.36	0.32
MON810	10.0	9.77	1.03	0.48

[a] Critical value of F is 3.02 (α=0.05)

Table 3. Summary of accuracy and precision statistics for the duplex real-time PCR method

		Trueness		Precision	Detection limit			Trueness		Precision	Detection limit
		Means	Bias					Means	Bias		
% (w/w)	Retained labs	GMO amount, %	True value, %	RSD_R[a], %	Below 20 copies[b]	% (w/w)	Retained labs	GMO amount, %	True value, %	RSD_R[a], %	Below 20 copies[b]
		GA21						P35S			
0.50	11	0.57	13.2	18	0/22	0.50	11	0.60	19.9	19	0/22
1.0	10	1.13	12.7	10	0/20	1.0	11	1.19	18.8	21	0/22
5.0	11	5.60	11.8	13	0/22	5.0	11	5.82	16.4	13	0/22
10.0	11	11.15	11.5	9.0	0/22	10.0	10	11.91	19.1	9.5	0/22

[a] RSD_R: Reproducibility relative standard deviation

[b] Below 20 copies refers to the ratio of the number of retained data below 20 cpies/the total number of retained data

consisting of three copies of its perfect gene cassette and three incomplete copies[*1]. In the whole recombinant insertion, five copies of the construct-specific segment of GA21 are supposed to be present (Fig. 1). We obtained the Cf values for the GA21 construct-specific method as between 1.40 and 2.01 in our previous studies[8)–10)]. The theoretically expected Cf value for the event-specific method would be one-fifth of these values, and thus would be between 0.28 and 0.40. Both of the Cf values determined for GA21 in this study were within this range.

Interlaboratory validation of the duplex real-time PCR method

After determination of the Cf values, the homogeneities of the blind samples were confirmed by one-way ANOVA. Ten tubes of each mixing sample were randomly selected twice. The DNA was then extracted from these samples, and quantitative PCR analyses of GA21 and MON810 were performed using each construct-specific method[8)–10)]. The measured copy numbers were converted into the GMO amount (%), and one-way ANOVA was then conducted on the data. The F- and p-values were calculated (Table 2). Even at the smallest value, 0.18, obtained by the MON810 specific quantification of the 0.50% sample, the p-values were larger than 0.05, indicating that the contents of both GA21 and MON810 of all the test samples were sufficiently homogeneous and met the requirements for the following interlaboratory study.

The developed duplex real-time PCR quantitative method was evaluated in a blind test performed by 11 laboratories using the AB 7900. The measurements of GA21 and P35S were carried out independently. A blank sample, with 0% GM content, was used to estimate invalid laboratories, and no laboratory was eliminated. All the submitted data except 0% were then handled according to the harmonized guidelines of AOAC[14)] to remove outlier laboratories with extreme variation using Cochran's test and with an extreme average level using Grubbs' test as previously described[9), 10)]. One Cochran outlier was detected in the 1.0% GA21 sample and one Grubbs outlier was detected in the 10.0% P35S sample. After removing these outliers, further statistical analyses were conducted. The trueness and precision were determined as the bias (mean-value, %) and reproducibility of relative standard deviation (RSD_R) for GA21 and P35S in individual samples (Table 3). The bias and RSD_R of GA21 were less than 15% and 20% in all samples, respectively. Both the bias and RSD_R of P35S were slightly higher than those of GA21, but were less than 20% and 25%, respectively. These obtained bias and RSD_R levels were simi-

[*1] Agbios database. http://www.cera-gmc.org/?action=gm_crop_database&

lar to or even less than those of previously reported GMO events[9), 10)]. In terms of the limit of quantitation (LOQ), all the measured copy numbers of the 0.5% samples were over 20 copies and there was no calibrant below 20 copies in this method. Therefore, we estimated that the LOQ for the GA21 event-specific and P 35S in the duplex PCR method was 0.50% or less.

The previously developed duplex real-time PCR method was validated in this interlaboratory study using AB 7900 instruments. The levels of obtained LOQ, trueness and precision were almost the same as those of other established methods[9), 10)] and the single laboratory evaluation[5)]. We thus consider that the duplex real-time PCR a good candidate for routine screening for GM maize commingled in agricultural crops.

Acknowledgements

We would like to thank the following collaborators for their participation in this study:

Applied Biosystems Japan, Tokyo, Japan
FASMAC, Kanagawa, Japan
Food and Agricultural Materials Inspection Center, Saitama, Japan
House Foods Co., Chiba, Japan
Japan Food Research Laboratories, Tokyo, Japan
Japan Frozen Foods Inspection Corporation, Kanagawa, Japan
Japan Grain Inspection Association, Tokyo, Japan
Japan Grassland Agriculture and Forage Seed Association, Tochigi, Japan
National Food Research Institute, Ibaraki, Japan
National Institute of Health Sciences, Tokyo, Japan
National Livestock Breeding Center, Nagano, Japan
Wakodo Co., Tokyo, Japan
Yokohama Plant Protection Station, Kanagawa, Japan

This work was supported by the Ministry of Agriculture, Forestry, and Fisheries of Japan Research Project, ("Assurance of Safe Use of Genetically Modified Organisms" and "Research Project for Genomics for Agricultural Innovation GAM-210") and by grants from the Food and Agricultural Materials Inspection Center project "Validation of PCR Methods of Quantitative and Qualitative Analysis for Genetically Modified Organisms" and the Ministry of Health, Labor and Welfare of Japan.

Reference

1) Akiyama, H., Nakamura, F., Yamada, C., Nakamura, K., Nakajima, O., Kawakami, H., Harikai, N., Furui, S., Kitta, K., Teshima, R. A screening method for the detection of the 35S promoter and the nopaline synthase terminator in genetically modified organisms in a real-time multiplex polymerase chain reaction using high-resolution melting-curve analysis. Biol. Pharm. Bull., 32, 1824–1829 (2009).
2) Randhawa, G. J., Chhabra, R., Singh, M. Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for the screening of genetically modified crops. J. Agric. Food Chem., 57, 5167–5172 (2009).
3) Grohmann, L., Brünen-Nieweler, C., Nemeth, A., Waiblinger, H. U. Collaborative trial validation studies of real-time PCR-based GMO screening methods for detection of the bar gene and the ctp2-cp4epsps construct. J. Agric. Food Chem., 57, 8913–8920 (2009).
4) Lu, I. J., Lin, C. H., Pan, T. M. Establishment of a system based on universal multiplex-PCR for screening genetically modified crops. Anal. Bioanal. Chem., 396, 2055–2064 (2010).
5) Oguchi, T., Onishi, M., Minegishi, Y., Kurosawa, Y., Kasahara, M., Akiyama, H., Teshima, R., Futo, S., Furui, S., Hino, A., Kitta, K. Development of quantitative duplex real-time PCR method for screening analysis of genetically modified maize. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan.), 50, 117–125 (2009).
6) Yoshimura, T., Kuribara, H., Matsuoka, T., Kodama, T., Iida, M., Watanabe, T., Akiyama, H., Maitani, T., Furui, S., Hino, A. Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. J. Agric. Food Chem., 53, 2052–2059 (2005).
7) Yoshimura, T., Kuribara, H., Kodama, T., Yamata, S., Futo, S., Watanabe, S., Aoki, N., Iizuka, T., Akiyama, H., Maitani, T., Naito, S., Hino, A. Comparative studies of the quantification of genetically modified organisms in foods processed from maize and soy using trial producing. J. Agric. Food Chem., 53, 2060–2069 (2005).
8) Kuribara, H., Shindo, Y., Matsuoka, T., Takubo, K., Futo, S., Aoki, N., Hirao, T., Akiyama, H., Goda, Y., Toyoda, M., Hino, A. Novel reference molecules for quantification of genetically modified maize and soybean. J. AOAC Int., 85, 1077–1089 (2002).
9) Shindo, Y., Kuribara, H., Matsuoka, T., Futo, S., Sawada, C., Shono, J., Akiyama, H, Goda, Y., Toyoda, M., Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. J. AOAC Int., 85, 1119–1126 (2002).
10) Kodama, T., Kuribara, H., Minegishi, Y., Futo, S., Watai, M., Sawada, C., Watanabe, T., Akiyama, H., Maitani, T., Teshima, R., Furui, S., Hino, A., Kitta, K. Evaluation of modified PCR quantitation of genetically modified maize and soybean using reference molecules: Interlaboratory study. J. AOAC Int., 92, 223–233 (2009).
11) Cochran, W. G. The distribution of the largest of a set of estimated variances as a fraction of their total. Annals of Eugenics, 11, 47–52 (1941).
12) Grubbs, F. E. Sample criteria for testing outlying observations. Ann. Math. Statist. Assn., 21, 27–58 (1950).
13) Grubbs, F. E. Procedures for detecting outlying observations in samples. Technometrics, 11, 1–21 (1969).
14) Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis. In Official Methods of Analysis of AOAC Int. 17th ed., volume II, Gaithersburg, MD, USA.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Practicable Group Testing Method to Evaluate Weight/Weight GMO Content in Maize Grains

Junichi Mano,[†] Yuka Yanaka,[†] Yoko Ikezu,[†] Mari Onishi,[‡] Satoshi Futo,[‡] Yasutaka Minegishi,[§] Kenji Ninomiya,[∥] Yuichi Yotsuyanagi,[∥] Frank Spiegelhalter,[⊥] Hiroshi Akiyama,[#] Reiko Teshima,[#] Akihiro Hino,[†] Shigehiro Naito,[†] Tomohiro Koiwa,[†] Reona Takabatake,[†] Satoshi Furui,[†] and Kazumi Kitta*[,†]

[†]National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12, Kannondai, Tsukuba, Ibaraki 305-8642, Japan

[‡]Fasmac Co., Ltd., 5-1-3, Midorigaoka, Atsugi, Kanagawa 243-0041 Japan

[§]Nippon Gene Co. Ltd., 1-5, Kandanishiki-cho, Chiyoda-ku, Tokyo 101-0054, Japan

[∥]Shimadzu Corporation, 1, Nishinokyo-Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan

[⊥]Genescan, Inc., 2315 N. Causeway Boulevard, Metairie, Louisiana 70001, United States

[#]National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Supporting Information

ABSTRACT: Because of the increasing use of maize hybrids with genetically modified (GM) stacked events, the established and commonly used bulk sample methods for PCR quantification of GM maize in non-GM maize are prone to overestimate the GM organism (GMO) content, compared to the actual weight/weight percentage of GM maize in the grain sample. As an alternative method, we designed and assessed a group testing strategy in which the GMO content is statistically evaluated based on qualitative analyses of multiple small pools, consisting of 20 maize kernels each. This approach enables the GMO content evaluation on a weight/weight basis, irrespective of the presence of stacked-event kernels. To enhance the method's user-friendliness in routine application, we devised an easy-to-use PCR-based qualitative analytical method comprising a sample preparation step in which 20 maize kernels are ground in a lysis buffer and a subsequent PCR assay in which the lysate is directly used as a DNA template. This method was validated in a multilaboratory collaborative trial.

KEYWORDS: GMO detection, detection, group testing, subsampling

INTRODUCTION

Industrial use of genetically modified organisms (GMOs) has been advancing, and many genetically modified (GM) crops have been put on the market in the past 15 years.[1] In maize, which is one of the four major GM crops, along with soybeans, cotton, and canola, stacked-event seeds, generated by crossing two or more single GM events, have been widely used.[1] Numerous safety assessments of GM crops and their derived foods and feeds have been conducted by authorities in countries around the world, and commercially available GM crops are considered to be as safe as their conventional (non-GM) counterparts. In many countries, however, the use of GM crops is controversial among general consumers, and the demand for conventional crops is deeply rooted. To expand consumers' choices, many countries have introduced legislation requiring labels to be applied to agricultural products that happen to contain approved GMOs at more than a certain threshold level. For example, the thresholds are set as 0.9%, 3%, and 5% in the European Union (EU), Korea, and Japan, respectively.[2] For products that do not carry GM labels, compliance with these regulations is checked at various points of the supply chain, often starting with the crops.

The regulations in some countries refer to the GM material in terms of weight/weight percentages, although the most commonly used technique for GMO quantification in grain is quantitative real-time polymerase chain reaction (PCR) analysis of bulk sample homogenates, and the analysis typically measures GMO contents based on the ratio of GM DNA to plant-species DNA. Because the GM stacked events contain the GM DNA corresponding to two or more single events, the GMO content of non-GMO maize samples with a small number of stacked-event kernels measured by real-time PCR leads to an overestimation as compared to the actual weight/weight GMO content.[3] In light of the increasing use of GM stacked events, it has become virtually impossible to accurately measure low-level GMO content on a weight/weight basis with current methodology. Recently, a single-kernel-based analytical system was developed and implemented in Japan as an official method, as one possible solution for the potential overestimation due to stacked events.[3−5] In this method, individual maize kernels are analyzed to determine their GM or non-GM status, and the weight/weight GMO content is evaluated based on the assumption that the ratio of GM kernels relative to the total number of kernels is equal to the weight/weight ratio.

Since Dorfman's report on blood testing for syphilis in 1943, the group testing strategy has been exploited in epidemiology,

Received: January 16, 2011
Accepted: May 23, 2011
Revised: April 4, 2011

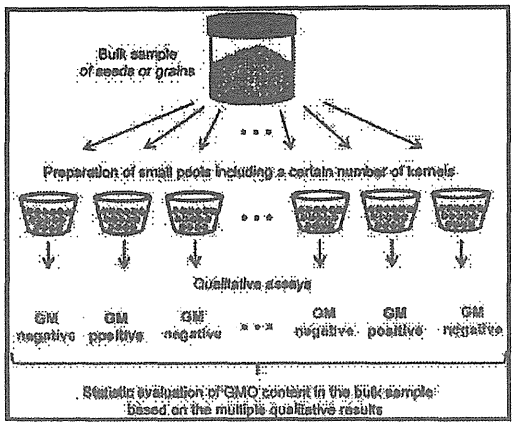

Figure 1. Group testing applied to GMO analysis.

genetics, blood-bank screening, drug discovery, biology, and plant pathology.[6,7] In this strategy, groups of units that make up an analytical sample are prepared. Then, qualitative analyses of multiple groups are individually performed, and the contents of the analyte are evaluated statistically.[8] When applied to GMO analysis of seeds or grains, each group contains a defined number of kernels from a larger bulk sample, and the GMO content is statistically evaluated based on qualitative results for multiple groups (Figure 1). Irrespective of the presence of stacked-event kernels, such a strategy enables the evaluation of GMO content on a weight/weight basis. Additionally, this strategy would be more efficient than a single-kernel-based strategy. In fact, the theoretical application of group testing strategy to GMO analysis has been investigated.[9−11] Meanwhile, to the best of our knowledge, a practical and accurate testing method to perform group testing for maize grains has not yet been reported. Accordingly, we present the development and validation of an efficient, easy-to-use PCR-based testing method for GMO detection in small pools of maize kernels.

MATERIALS AND METHODS

Cereal Materials. The representative GM maize events used were Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507, DAS59122, MON88017, and MIR604. F1-generation seeds of Bt11 and Event176 and ground F1-generation seeds of GA21 and MIR604 were kindly provided by Syngenta Seeds (Basel, Switzerland). F1-generation seeds of MON810, MON863, NK603, and MON88017 were kindly provided by Monsanto (St. Louis, MO, USA), and F1-generation seeds of TC1507 and DAS59122 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). F1-generation seeds of T25 were imported directly from the United States. Five conventional maize seeds were used as non-GM maize: DKS37 and RX740 maize from Monsanto; QC9651 maize from Quality Technology International (Huntley, IL, USA); and Strike5512 and LG2265 maize, obtained in Japan. Dry conventional soybean seeds directly imported from the United States were used as non-GM soy. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan.

Preparation of Genomic DNAs. For the preparation of purified DNA extracts, all dry seeds were ground with a P-14 speed rotor mill (Fritsch, Idar-Oberstein, Germany). For maize, soy, wheat, barley, and rice, DNA extraction was performed using a DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) as described previously.[13] The DNA concentration of solutions was determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). DNA concentration was calculated with 1 optical density unit at 260 nm equal to 50 ng/μL. All extracted DNAs were diluted to 20 ng/μL with sterile distilled water. Genomic DNAs were analyzed using a real-time PCR array system as previously reported,[14] and the purity of the samples was confirmed.

Preparation of Plasmid DNAs. To establish the method for group testing, we developed two duplex real-time PCR assays: a GM maize screening assay and an experimental control assay. The GM maize screening assay was designed to detect the 35S promoter region (P35S) and NOS terminator region (TNOS) widely introduced into commercially available GM maize events. The experimental control assay was intended to detect both the starch synthase IIb gene derived from *Zea mays* (SSIIb) as the endogenous reference DNA and an artificial sequence on the pART plasmid as an internal positive control (IPC). The pUC19 plasmids harboring each of the target sequences, namely, P35S, TNOS, SSIIb, and IPC, were prepared after cloning in *Escherichia coli* DH5α and are denoted pP35S, pTNOS, pSSIIb, and pART, respectively. The target sequences were confirmed to be single and correct by nucleotide sequence analyses. The sequence information is included in the Supporting Information. The plasmids were purified by cesium chloride/ethidium bromide equilibrium centrifugation[15] and then diluted to the given concentration with 5 ng/μL ColE1 plasmid solution in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (Nippon Gene, Tokyo, Japan).

PCR Assays. The two duplex real-time PCR assays, GM maize screening and experimental control assays, were developed as described above. The reaction mixture for the GM maize screening assay consisted of 12.5 pmol of P35S 1-5′ (5′-ATTGATGTGATATCTCCACTGACGT-3′), P35S 1-3′ (5′-CCTCTCCAAATGAAATGAACTTCCT-3′), TNOS 2-5′ (5′-GTCTTGCGATGATTATCATATAATTTCTG-3′), and TNOS 2-3′ (5′-CGCTATATTTTGTTTTCTATCGCGT-3′) primers; 2.5 pmol of P35S-Taq (5′-CCCACTATCCTTCGCAAGACCCTTCCT-3′) and TNOS-Taq (5′-AGATGGGTTTTTATGATTAGAGTCCCGCAA-3′) probes; 2.5 μL of DNA template; 0.5 μL of ROX Reference Dye (Life Technologies); 0.625 units of BIOTAQ HS DNA polymerase (Shimadzu, Kyoto, Japan); and 12.5 μL of 2 × Ampdirect Plus buffer (Shimadzu) in a total volume of 25 μL. The reaction mixture for the experimental control assay consisted of 12.5 pmol of IPC 1-5′ (5′-CCGAGCTTACAAGGCAGGTT-3′), IPC 1-3′ (5′-TGGCTCGTACACCAGCATACTAG-3′), SSIIb 1-5′ (5′-CTCCCAATCCTTTGACATCTGC-3′), and SSIIb 1-3′ (5′-TCGATTTCTCTCTTGGTGACAGG-3′) primers; 2.5 pmol of IPC 1-Taq (5′-TAGCTTCAAGCATCTGGCTGTCGGC-3′) and SSIIb-Taq (5′-AGCAAAGTCAGAGCGCTGCAATGCA-3′) probes; 40 theoretical copies of the pART plasmid; 2.5 μL of DNA template; 0.5 μL of ROX Reference Dye, 0.625 units of BIOTAQ HS DNA polymerase; and 12.5 μL of 2 × Ampdirect Plus in total volume of 25 μL. The oligonucleotide DNAs for PCR primers and TaqMan probes were synthesized by Fasmac (Atsugi, Japan) and Biosearch Technologies (Novato, CA, USA), respectively. P35S-Taq, TNOS-Taq, and IPC 1-Taq were labeled with 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ1) dyes at the 5′ and 3′ terminals, respectively. For SSIIb-Taq, hexachlorofluorescein (HEX) was used in place of FAM dye. Thermal cycling of reaction mixtures was carried out with a 7900HT real-time PCR instrument (Life Technologies, Carlsbad, CA, USA) unless otherwise specified. The thermal cycling condition was set as 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 65 °C under 9600 emulation mode. Data analysis was performed using Sequence Detection Software, version 2.3. The manual Ct mode (threshold, 0.256 for FAM and 0.064 for HEX) and manual baseline mode (start of baseline, 3; end of baseline, 15)

were set at the "Delta Rn vs. Cycle" view of the "Amplification Plot" feature. DNA amplifications with threshold-cycle (Ct) values below 40 were determined to be positive. Concerning PCR assays performed with a 7500 real-time PCR instrument (Life Technologies), all experiments were carried out as described above, except that the volume of ROX reference dye was set as 0.05 μL and Sequence Detection Software, version 1.4, was used for data analysis.

Design of Testing Protocol. We designed the following testing protocol:

Step 1. Groups comprising 20 maize kernels each are prepared using a grain counter plate on which only 20 holes are available (For 100 Soybeans; Fuji Kinzoku, Tokyo, Japan), and they are put into glass vessels with a capacity of 75 mL for use with a Milser 800-DG household food processor (Iwatani, Tokyo, Japan). The number of groups in one experiment is variable depending on the analyst's purpose.

Step 2. Twenty milliliters of a lysis buffer is added to each glass vessel. One liter of the lysis buffer contains of 20 mL of 1 mol/L Tris-HCl buffer solution (Nacalai Tesque, Kyoto, Japan), 10 mL of 0.5 mol/L EDTA solution (Nacalai Tesque), 80 mL of 5 mol/L sodium chloride (NaCl) solution (Nacalai Tesque), and 30 mL of 10% sodium dodecyl sulfate (SDS) solution (Nacalai Tesque) in distilled water. Each group is ground for 20 s with the household food processor. After 10 min of incubation at room temperature, the lysate in each glass vessel is vigorously shaken by hand. After 10 min of static standing to allow solid–liquid separation, 50 μL of the supernatant is moved to a plastic tube. Each portion of supernatant is diluted 2-fold with sterile distilled water. The diluted solution is centrifuged at more than 1000g on a personal benchtop centrifuge for 1 min and then used for the following PCR assay.

Step 3. PCR mixtures are prepared with the supernatant for both GM maize screening and experimental control assays, and thermal cycling is performed as described above.

Step 4. The data from real-time PCR are analyzed with Sequence Detection Software as described in the PCR Assays section. If SSIIb or IPC detection is determined to be negative by the experimental control assay, the group in question is rejected. If both SSIIb and IPC are positive, the group is determined to be either GM-positive or GM-negative based on the result of the GM maize screening assay.

Evaluation of the Testing Protocol. To evaluate the linearity of the PCR assays, we analyzed the respective plasmid DNAs with theoretical numbers of copies of 250000, 20000, 1500, 125, and 20 (n = 3). Then, we calculated the regression lines from the averages of triplicate PCR results. To evaluate the specificity of the PCR assays, we analyzed the genomic DNAs derived from various kinds of GM events and plant materials using both assays (n = 6). Ct values of P35S/TNOS detection by the GM maize screening assay and those of SSIIb and IPC detection by the experimental control assay were measured. To evaluate the sensitivity of the PCR assays, we analyzed plasmid DNAs with low theoretical numbers of copies (40, 20, 10, and 0; n = 21 for each dilution level) and counted the number of positive results. For the comparative analysis of PCR inhibition between P35S, TNOS, and IPC detections, we selected SDS, NaCl, EDTA, and crude maize extracts as PCR inhibitors. Under the coexistence of these possible inhibitors at various concentrations, positive and negative detections were examined both in the GM maize screening assay with 40 copies of the pP35S or pTNOS plasmid and in the experimental control assay with 40 copies of the pART plasmid. For the preparation of the crude maize extract, 1 g of the ground DK537 maize sample was incubated for 1 h with vigorous shaking at room temperature in 3 mL of the lysis buffer. After centrifugation at 15000g for 1 min, the supernatant was used as the crude maize extract.

We prepared simulated groups, each of which consisted of 19 kernels of non-GM maize and one MON810 kernel. As non-GM materials, DK537, RX740, QC9651, LG2265, and Strike5512 maize were individually used. The simulated groups were analyzed in accordance with the testing protocol (n = 6 for each material).

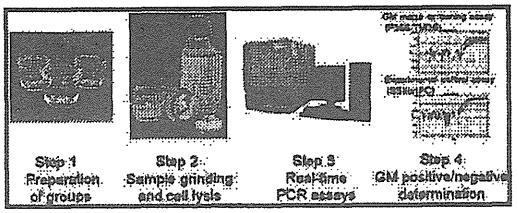

Figure 2. Overview of the testing protocol.

Collaborative Trial for Method Validation. For the collaborative trial, DK537 maize and F1-generation seeds of MON810 maize were used as non-GM and GM maize materials, respectively. All MON810 kernels were cut in half with a knife to inhibit germination, and simultaneously, approximately 2-mg fragments were scraped off individual half-cut GM kernels. To check for an adventitious presence of non-GM kernels in the GM seed lot, we suspended these fragments in 50 μL of the lysis buffer with sterile toothpicks and then analyzed them according to the testing protocol beginning with the 10-min incubation in step 2. We confirmed GM-positive detection for each MON810 kernel. The AOAC guideline specifies 10 laboratories reporting 2 analyte levels per matrix, 6 test samples per level, and 6 negative controls per matrix as the minimum criteria for the validation of qualitative methods.[16] These criteria also satisfy the requirements outlined in McClure's report.[17] Accordingly, we prepared groups consisting of 2 GM kernels and 18 non-GM kernels, groups consisting of 1 GM kernel and 19 non-GM kernels, and groups consisting of 20 non-GM kernels. These groups were named A, B, and C groups, respectively. As a set of blind samples for a laboratory, 6 A groups, 6 B groups, and 6 C groups were sorted at random and numbered from 1 to 18. A set of blind samples was provided to each of 12 laboratories. In each laboratory, the blind samples were analyzed in one experiment according to the testing protocol.

RESULTS AND DISCUSSION

Design of the Testing Protocol. To perform group testing for maize grains in a practical manner, it is essential to develop an easy-to-use, high-throughput, and cost-effective sample pretreatment and GM maize screening assay for groups of kernels. A series of immunoassays for individual GM traits might be a good candidate, but a protein-based methodology limits the range of detectable GM events. We designed a testing method comprising a sample pretreatment step in which a group of maize kernels is ground in a lysis buffer with a household food processor and a subsequent PCR assay step in which the lysed sample is directly analyzed as a DNA template. We experimentally adjusted testing conditions, and the testing protocol was fixed as described in the Materials and Methods section. An overview of the testing protocol is shown in Figure 2. In this testing protocol, we fixed the number of kernels in a group to 20. This was because our preliminary investigation indicated that a group consisting of 20 kernels was easy to handle and gave stable PCR assay results. For sample preparation, we recommend using a grain counter plate to make small pools efficiently without intentional bias. For the sample pretreatment step, we used a household food processor that permitted sequential grinding of many samples just by changing glass vessels. The use of a household food processor promises a high-throughput treatment with a

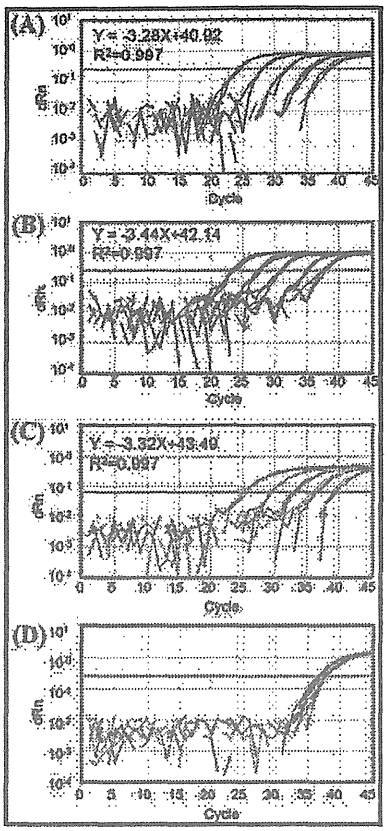

Figure 3. DNA amplification lines and parameters of their regression lines. Dilution series of plasmid DNAs were analyzed in triplicate. (A) pP35S series in the GM maize screening assay, (B) pTNOS series in the GM maize screening assay, (C) pSSIIb series in the experimental control assay, and (D) IPC detection results in the experimental control assay with the pSSIIb series. Regression lines were calculated from the mean values of triplicate analyses, and their parameters are shown in plots A–C.

minimum investment for grinding instruments. In addition, sample grinding in a lysis buffer does not require handling of dry flour, which simplifies the method and reduces the chance of contamination. For the PCR assay step, we designed two qualitative duplex real-time PCR assays, namely, GM maize screening and experimental control assays, using Ampdirect technology as a PCR reagent, which reduces the influence of PCR inhibitors. For the GM maize screening assay, the P35S and TNOS regions were selected as targets, because commercially available GM events have at least one, if not both, of these regions as part of their recombinant DNAs. TaqMan probes both of P35S and TNOS were labeled with FAM as a reporter dye, because P35S and TNOS detections were not necessarily distinguished. Meanwhile, we developed an experimental control assay to individually detect both SSIIb and 40 copies of pART, as the endogenous reference DNA and as an IPC, respectively. TaqMan probes for SSIIb and IPC detections were labeled with HEX and FAM, allowing us to distinguish between SSIIb and IPC. The experimental control assay was designed to confirm that the reaction mixture contained sufficient DNA extraction without PCR inhibition. To avoid detecting very tiny amounts of contamination, in terms of analytical robustness, we decided that DNA amplifications with Ct values of up to 40 were positive.

Evaluation of PCR Assays. We evaluated analytical performances of the PCR assay step. To do so, we prepared plasmid DNAs, each of which had a single target sequence for P35S, TNOS, SSIIb, and IPC detection. We confirmed the amplification linearity by using dilution series of plasmids except for pART (Figure 3). Detection results for P35S, TNOS, and SSIIb showed high coefficient values (>0.990). IPC detections were successfully obtained, irrespective of predominant SSIIb amplification in the same reaction mixture. Then, we confirmed the detection specificity with genomic DNAs from commercially distributed GM maize events and non-GM crops (Figure 4). P35S and/or TNOS regions were detected for all GM maize events, and these Ct values roughly corresponded to the numbers of copies of the P35S and/or TNOS regions in each event. Meanwhile, for non-GM maize, soy, wheat, barley, and rice, nonspecific detection was not observed, as expected. Although the specificity evaluation was carried out using only the single-GM-event samples, the results suggested that the GM stacked events derived from the single events would be detected in the developed assays. By analyzing the plasmid dilution series, we confirmed that the detection sensitivity of our method was high enough to detect 40 copies of target DNAs (Table 1). Then, we compared PCR inhibitions between P35S, TNOS, and IPC detections. We selected SDS, NaCl, EDTA, and crude maize extracts as PCR inhibitors that exist in PCR mixtures. Under the coexistence of these inhibitors at various concentrations, positive and negative detections were counted both in the GM maize screening assay with the pP35S or pTNOS plasmid and in the experimental control assay with the pART plasmid (Table 2). The results indicated that the IPC detection was sensitive to PCR inhibitors as well as P35S and TNOS detections when at least 40 copies of the P35S or TNOS regions were included in a reaction mixture. We also evaluated the PCR assays on the 7500 real-time PCR instrument, and the results are available in the Supporting Information. There were no large differences in results between the 7900HT and 7500 real-time PCR instruments.

Analysis of Simulated Samples According to the Testing Protocol. We performed analyses with the simulated groups of a maize sample that included one GM kernel among 20 kernels (Figure 5). As the GM maize kernel, we used F1-generation seeds of the *MON810* event, which has the lowest number of copies of the target of the GM maize screening assay. No false negative result was observed in the GM maize screening assay, suggesting that the testing protocol had the capacity to detect at least one GM kernel in a group. Homogeneity of Ct-value variances of SSIIb detection between different non-GM materials was confirmed by Bartlett's test ($\alpha = 0.05$), and one-way analysis of variance (ANOVA) ($\alpha = 0.05$) of the data showed no significant differences ($p = 0.08$). This indicated that stable DNA extraction was achieved, irrespective of maize materials. In these results, the numbers of copies of SSIIb from 20 kernels were calculated as being between 5200 and 21000 copies (mean value of all results, 15000 copies) based on the calibration curve of the pSSIIb plasmid dilution series. This suggested that, if there was one kernel of GM maize in a group, at lease 5200/20 copies of P35S and/or TNOS regions would be expected to be present in a

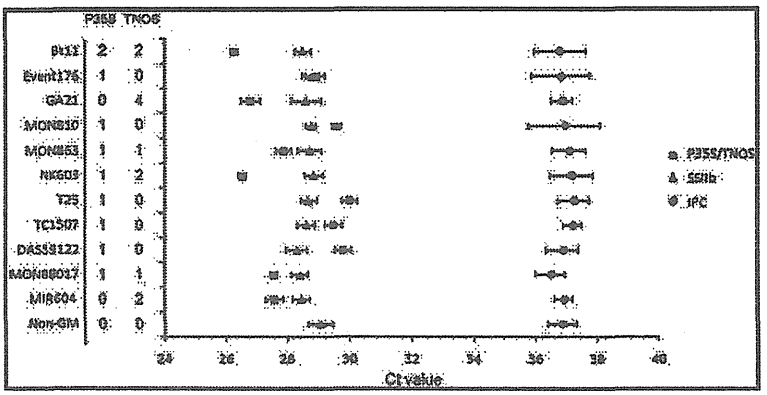

Figure 4. Specificity evaluation of PCR assays. DNA samples from GM events and non-GM maize were subjected to PCR assays ($n = 6$). The means of Ct values ± standard deviations are shown in the graphs. The numbers of P35S and TNOS regions in each GM-event haploid genome are summarized beside the names of the GM events.

Table 1. Sensitivity Evaluation of PCR Assays

detection	theoretical number of plasmid copies	number of positives	positive rate (%)
P35S	40	21/21	100
	20	21/21	100
	10	16/21	76
	0	0/21	0
TNOS	40	21/21	100
	20	21/21	100
	10	20/21	95
	0	0/21	0
SSIIb	40	21/21	100
	20	18/21	86
	10	12/21	57
	0	0/21	0

Table 2. Comparative Analyses of PCR Inhibition between IPC, P35S, and TNOS Detections

inhibitor	final concentration	IPC detection	P35S detection	TNOS detection
SDS	0.01%	3/3	3/3	3/3
	0.05%	3/3	3/3	3/3
	0.5%	0/3	0/3	0/3
NaCl	2 mM	3/3	3/3	3/3
	10 mM	3/3	3/3	3/3
	100 mM	0/3	0/3	0/3
EDTA	0.1 mM	3/3	3/3	3/3
	0.2 mM	3/3	3/3	3/3
	1 mM	0/3	0/3	0/3
crude maize extract	1/1000	3/3	3/3	3/3
	1/500	2/3	3/3	3/3
	1/10	0/3	0/3	0/3

reaction mixture, and thus the IPC detection designed to have 40 copies of plasmid DNA as a template would be capable of checking PCR inhibition in the GM maize screening assay.

Evaluation of Robustness in the Sample Pretreatment Step. We evaluated the robustness of the sample pretreatment step with groups of non-GM kernels by slightly changing pretreatment conditions ($n = 6$ per condition). The modified conditions were the grinding time (10 s, 15 s, 20 s, or 25 s), the lysis time (5 min, 10 min, or 20 min), and the lysis temperature (15, 20, or 25 °C). The Ct values of SSIIb and IPC detections in the experimental control assay were evaluated (Figure 6). The homogeneity of Ct-value variances between conditions was confirmed by Bartlett's test ($\alpha = 0.05$), and then Ct values were analyzed by one-way ANOVA ($\alpha = 0.05$). Ct values under various lysis times and temperatures did not significantly differ. Meanwhile, Ct values of SSIIb detection under the various grinding times showed significant differences, suggesting that the amount of extracted DNA was influenced by the grinding time. We concluded that the sample pretreatment step was sufficiently robust in terms of the lysis time and temperature, but that the grinding time should be strictly controlled.

Collaborative Trial for Method Validation. A collaborative trial was carried out according to the procedure described in a previous report and guideline.[16,17] The results for the individual groups are available in the Supporting Information. All of the results showed the expected positive/negative determinations corresponding to the presence/absence of GM kernel(s) in each group, except for a result that was rejected because of unsuccessful IPC detection. The results indicated that the method accurately detected the presence of GM and the absence of cross-contamination between groups. After removal of the result rejected because of the unsuccessful IPC detection, the false-negative rates were calculated separately for the A and B groups, and the false-positive rate was calculated for the C groups (Table 3). Both false-negative rates were 0%, which fulfilled the criterion for the limit of detection for qualitative GMO detection methods as described in the ISO standard regarding GMO analysis.[18] In addition, the Ct values of the detections were found to be stable even under inter-laboratory evaluation (Figure 7). Thus, the testing method was validated to have sufficient performance for the reliable detection of one GM maize kernel in a group.

dx.doi.org/10.1021/jf200212v |J. Agric. Food Chem. XXXX, XXX, 000–000

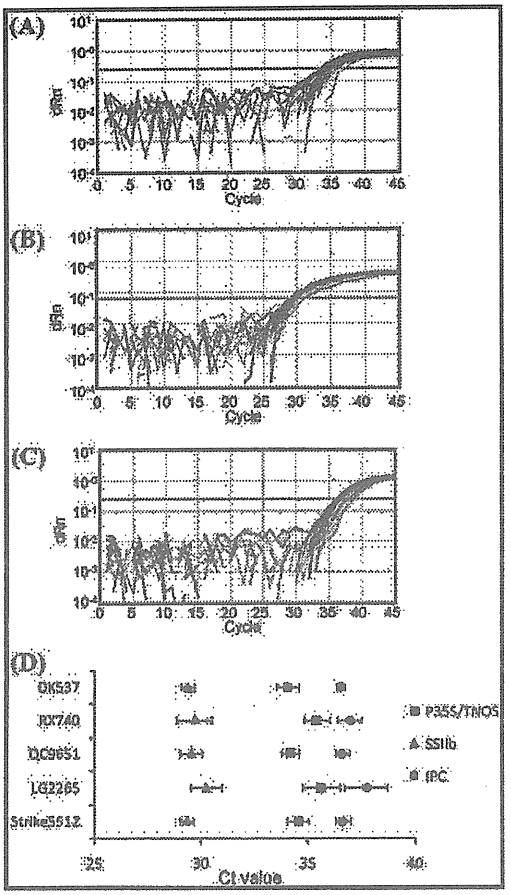

Figure 5. Testing results for the simulated samples including a MON810 kernel mixed in with five kinds of non-GM maize materials. (A) Amplification lines in the GM maize screening assay, (B) amplification lines of SSIIb detection in the experimental control assay, (C) amplification lines of IPC detection in the experimental control assay, and (D) summary of Ct value data for each non-GM background (means ± standard deviations, $n = 6$).

Practical Use of the Group Testing Based on the Developed Method. In summary, we have described an easy-to-use analytical method for group testing. This method was efficient enough to analyze 18 groups within 3 h at a low cost. Although our method harnesses two targets, namely, P35S and TNOS, to cover the commercially distributed GM maize events so far, this might become insufficient as new GMO events become available. There have been some reports describing highly multiplexing real-time PCRs for qualitative GMO detection.[19−22] The availability of the PCRs described in these studies suggests that it will be possible to update our method to test for new GMOs as they enter the market.

To practice group testing, it is indispensable to first determine the appropriate testing conditions, that is, the number of kernels per group (group size), the number of groups, and the maximum

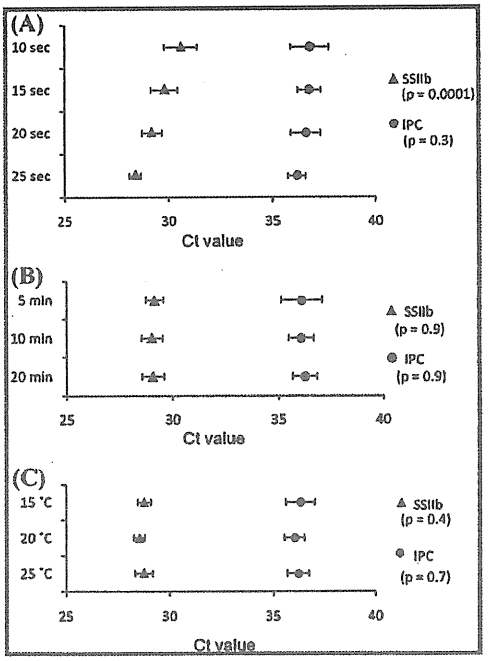

Figure 6. Robustness evaluation for the pretreatment step by simulated sample analyses. Effects by (A) grinding time, (B) lysis time, and (C) lysis temperature. Data are shown as means of Ct values ± standard deviations ($n = 6$). p values given by one-way ANOVA ($\alpha = 0.05$) are shown under the graph legends.

Table 3. Summary of Results in the Interlaboratory Study

group	number of rejections	false-positive results	false-positive rate (%)	false-negative results	false-negative rate (%)
A	1/72	–	–	0/71	0
B	0/72	–	–	0/72	0
C	0/72	0/72	0	–	–

number of GM-positive groups for acceptance. Statistical calculation programs previously reported, such as Seedcalc, facilitate the determination of the optimal testing conditions depending on the analyst's purpose.[9,12] In our method, the group size was fixed at 20; however, the other parameters could be freely chosen. We confirmed that, even when the group size was fixed at 20, the testing conditions suitable for various threshold levels of GMO content such as 0.9%, 3%, and 5% could be selected by using the already existing calculation programs.

As an official method in Japan, the single-kernel-based method has already been used to determine whether the GMO content in a bulk maize sample exceeds 5%. The testing procedure requires analysis of 90 kernels for the first screening. If there are 3 or more GM kernels in the first 90 kernels tested, another set of 90 kernels must be tested. If the total number of GM kernels in the two tests (180 kernels) is 9 or less, then the GMO content of the bulk

dx.doi.org/10.1021/jf200212v |J. Agric. Food Chem. XXXX, XXX, 000–000

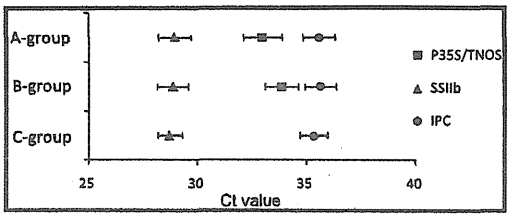

Figure 7. Summary of Ct value data in the interlaboratory study. The definitions of the groups A–C are given in the Materials and Methods section. Data are shown as the means ± standard deviations (n = 71 for the A group and n = 72 for the B and C groups).

sample is below 5% and is acceptable.[23] Based on the operating characteristic curve calculated by the Seedcalc program, we can design group testing that has approximately the same accuracy of judgment as the single-kernel-based method. The designed sampling plan is as follows: A group contains 20 maize kernels, and 10 groups are analyzed for the first screening. If there are 7 or more GM-positive groups in the first screening, another set of 10 groups will be tested. If the total number of GM-positive groups in the two tests (20 groups) combined is 12 or less, the GMO content of the bulk sample is determined to be below 5%. A comparison of operating characteristic curves between the single-kernel-based method and our group testing is provided in the Supporting Information. The slope of an operating characteristic curve represents the uncertainty of judgment that is caused by the sampling, and an analyst should take it into consideration. The introduction of group testing using our method will significantly decrease time and cost for inspection.

Furthermore, calculation programs permit the estimation of a GMO content value with confidence intervals from the testing results. For example, when 8 groups are determined as GM-positive in the testing of 20 groups containing 20 kernels per group, the GMO content will be estimated as 2.52% and its two-sided confidence interval will be between 1.06% and 4.97% at the 95% confidence level. In this manner, one can obtain quantitative information on the GMO content of the bulk sample based on the qualitative testing results and the established statistics.

We believe that group testing is a useful measure for weight/weight GMO content evaluation in maize grains, irrespective of increasing GM stacked events. Certainly, the strategy limits a sample to only seeds or grains and is not applicable to processed foods. However, group testing would contribute to the assured segregation of GM and non-GM maize through the production and transportation systems.

■ ASSOCIATED CONTENT

Supporting Information. Nucleotide sequence information on PCR amplicons, results of analytical performance evaluation on a 7500 real-time PCR instrument, results of interlaboratory studies, and comparative analysis of the single-kernel-based method and the designed group testing. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: +81-29-838-7369. Fax: +81-29-838-7369. E-mail: kaz@affrc.go.jp.

■ ACKNOWLEDGMENT

We thank Monsanto, Pioneer Hi-Bred International, and Syngenta Seeds for providing plant materials. We thank the following participants of the interlaboratory study: Calbee Foods (Tokyo, Japan), Food and Agricultural Materials Inspection Center (Saitama, Japan), Fasmac (Atsugi, Japan), Japan Food Research Laboratories (Tokyo, Japan), Japan Frozen Foods Inspection Corporation (Tokyo, Japan), Japan Grassland Agriculture and Forage Seed Association (Tokyo, Japan), Japan Inspection Association of Food and Food Industry Environment (Tokyo, Japan), National Center for Seeds and Seedlings (Tsukuba, Japan), National Food Research Institute (Tsukuba, Japan), National Institute of Health Sciences (Tokyo, Japan), National Livestock Breeding Center (Saigo, Japan), and Plant Protection Station of MAFF (Yokohama, Japan). This research was funded by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan ("Assurance of Safe Use of Genetically Modified Organisms" and "Research Project for Genomics for Agricultural Innovation GAM-211") and by a grant from the Ministry of Health, Labor and Welfare of Japan.

■ ABBREVIATIONS USED

ANOVA, analysis of variance; BHQ1, black hole quencher 1; EDTA, ethylenediaminetetraacetic acid; EU, European Union; GM, genetically modified; GMO, genetically modified organism; HEX, hexachlorofluorescein; IPC, internal positive control; PCR, polymerase chain reaction; NaCl, sodium chloride; SDS, sodium dodecyl sulfate; TNOS, NOS terminator region; SSIIb, starch synthase IIb gene of *Zea mays*; P35S, 35S promoter region; Ct, threshold cycle; UV, ultraviolet; FAM, 6-carboxyfluorescein.

■ REFERENCES

(1) James, C. *Executive Summary—Global Status of Commercialized Biotech/GM Crops: 2009*; ISAAA Brief 41-2009; International Service for the Acquisition of Agri-Biotech Applications (ISAAA): Ithaca, NY, 2009.

(2) Davison, J.; Bertheau, Y. *Cereal Foods World* 2008, *53*, 186–196.

(3) Akiyama, H.; Sakata, K.; Kondo, K.; Tanaka, A.; Liu, M. S.; Oguchi, T.; Furui, S.; Kitta, K.; Hino, A.; Teshima, R. *J. Agric. Food Chem.* 2008, *56*, 1977–1983.

(4) Notice No. 0803, Article 8; Department of Food Safety, Ministry of Health, Labour and Welfare of Japan: Tokyo, Japan, 2009.

(5) Akiyama, H.; Watanabe, T.; Wakabayashi, K.; Nakade, S.; Yasui, S.; Sakata, K.; Chiba, R.; Spiegelhalter, F.; Hino, A.; Maitani, T. *Anal. Chem.* 2005, *77*, 7421–7428.

(6) Dorfman, R. *Ann. Math. Stat.* 1943, *14*, 436–440.

(7) Bilder, C. R.; Tebbs, J. M. *J. Stat. Comput. Simul.* 2009, *1*, 67–80.

(8) Chen, C. L.; Swallow, W. H. *Biometrics* 1990, *46*, 1035–1045.

(9) Remund, K. M.; Dixon, D. A.; Wright, D. L.; Holden, L. R. *Seed Sci. Res.* 2001, *11*, 101–119.

(10) Kobilinsky, A.; Bertheau, Y. *Chemom. Intell. Lab. Syst.* 2005, *75*, 189–200.

(11) Laffont, J. L.; Remund, K. M.; Wright, D.; Simpson, R. D.; Grégoire, S. *Seed Sci. Res.* 2005, *15*, 197–204.

(12) Yamamura, K.; Hino, A. *Commun. Stat. Simulat.* 2007, *36*, 949–957.

(13) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. *J. AOAC Int.* 2002, *85*, 1077–1089.

(14) Mano, J.; Shigemitsu, N.; Futo, S.; Akiyama, H.; Teshima, R.; Hino, A.; Furui, S.; Kitta, K. *J. Agric. Food Chem.* 2009, *57*, 26–37.

(15) Nakayama, H.; Nishikata, T. *Bio-jikken irasutoreiteddo*; Shujun-sha, Shinagawa-ku: Tokyo, Japan, 1995; Vol. 2, 24–30 (in Japanese).

(16) AOAC International. Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis. In *Official Methods of Analysis of AOAC International*, 17th ed.; AOAC International: Gaithersburg, MD, 2003; Volume II.

(17) McClure, F. D. *J. AOAC Int.* **1990**, *73*, 953–960.

(18) *Foodstuffs—Methods of analysis for the detection of genetically modified organisms and derived products—General requirements and definitions*; ISO 24276; International Organization for Standardization (ISO): Geneva, Switzerland, 2006.

(19) Waiblinger, H. U.; Ernst, B.; Anderson, A.; Pietsch, K. *Eur. Food Res. Technol.* **2008**, *226*, 1221–1228.

(20) Gaudron, T.; Peters, C.; Boland, E.; Steinmetz, A.; Moris, G. *Eur. Food Res. Technol.* **2009**, *229*, 295–305.

(21) Dörries, H. H.; Remus, I.; Grönewald, A.; Grönewald, C.; Berghof-Jäger, K. *Anal. Bioanal. Chem.* **2010**, *396*, 2043–2054.

(22) Bahrdt, C.; Krech, A. B.; Wurz, A.; Wulff, D. *Anal. Bioanal. Chem.* **2010**, *396*, 2103–2112.

(23) Akiyama, H.; Sakata, K.; Kondo, K.; Tanaka, A.; Liu, M. S.; Oguchi, T.; Furui, S.; Kitta, K.; Hino, A.; Teshima, R. *J. Agric. Food Chem.* **2008**, *56*, 1977–1983.

dx.doi.org/10.1021/jf200212v |*J. Agric. Food Chem.* XXXX, XXX, 000–000

ノ ー ト
Note

日本食品化学学会誌、Vol. 19(3), 215-222(2012)
Japanese Journal of Food Chemistry and Safety (JJFCS)

プラスミドDNAを用いた中国産安全性未承認遺伝子組換えコメ検査に関する外部精度管理調査

(2012年5月22日受付)
(2012年11月1日受理)

笠間菊子[a)]、井上雪乃[a)]、穐山 浩[b)]、鈴木達也[a)]、坂田こずえ[b)]、
中村公亮[b)]、大島赴夫[a)]、小島幸一[a)]、近藤一成[b)]、手島玲子[b)]

a) 財団法人 食品薬品安全センター秦野研究所
b) 国立医薬品食品衛生研究所

Proficiency testing of unauthorized genetically modified rice using plasmid DNA test samples

(Received May 22, 2012)
(Accepted November 1, 2012)

Kikuko Kasama[a)], Yukino Inoue[a)], Hiroshi Akiyama[b)], Tatsuya Suzuki[a)], Kozue Sakata[b)], Kosuke Nakamura[b)], Yukio Ohshima[a)], Koichi Kojima[a)], Kazunari Kondo[b)], Reiko Teshima[b)]

a) Hatano Research Institute, Food and Drug Safety Center
b) National Institute of Health Sciences

Abstract

Proficiency testing of the assay for detecting unauthorized genetically modified (GM) rice was conducted. Test samples prepared from genomic DNA extracted from non-GM rice and two types of positive control plasmid DNA for qualitative PCR test and for real-time PCR test were sent to 33 laboratories for the purpose of detection using the Japanese official method. The test samples for qualitative PCR testing were prepared to be 1 v/v % and 0.05 v/v % in the ratio of the plasmid DNA for qualitative PCR to genomic DNA, and the test samples for real-time PCR testing were prepared to be 0.6 v/v % and 0.12 v/v % in the ratio of the plasmid DNA for real-time PCR to genomic DNA. For the proficiency testing, 31 of 33 laboratories correctly identified all samples. However, two laboratories reported incorrect identification of the non-GM samples. We considered that the incorrect results were due to contaminations of the reaction mixture with plasmid DNA or other interfering DNA. For the real-time PCR analysis, some laboratories using the specific real-time PCR device reported elevated baseline in the multicomponent analysis. The results of this proficiency testing study suggest that the proficiency testing using plasmid DNA samples was useful tool to investigate important factors affecting the reliability of detection results of unauthorized genetically modified rice.

Keywords : 遺伝子組換えコメ、プラスミドDNA、外部精度管理調査
genetically modified rice, plasmid DNA, proficiency testing

I 緒言

安全性審査を受けていない遺伝子組換え(GM)食品は、食品衛生法に基づいて厚生労働大臣が定めた「食品、添加物等の規格基準」により製造、輸入、販売等が禁止されている。

2006年9月、環境保護団体グリーンピースがフランスとドイツで、また同じくFriends of the earthがイギリスで、中国産のコメ加工品から、GMコメを検出したと発表した[*1, *2]。日本では今のところ安全性審査を完了したGMコメは存在しないため、GMコメを含む食品は直ちに流通禁止となる。このため2006年9月26日、厚生労働省医薬食品安全部監視安

連絡先：〒158-8501 東京都世田谷区上用賀1-18-1 国立医薬品食品衛生研究所 食品添加物部 穐山 浩
Corresponding author: Hiroshi Akiyama, Division of Food Additives, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

*1 Illegal genetically engineered rice from China in European rice products http://www.greenpeace.org/international/press/repors/IllegalChinaGErice

*2 Illegal GM rice found in UK. http://www.foe.co.uk/resource/press_releases/illegal_gm_rice_fonud_in_u_05092006.html

全課輸入食品安全対策室は、事務連絡 *3 で中国産 GM コメに特異的な検査方法を定め、中国産コメ加工品のモニタリング検査頻度を 50% に引き上げた。その後、2007 年 1 月 26 日付けプレスリリース *4 により、わが国でもこの時点で 6 件の安全性未審査の GM コメの検出事例があったことが報告された。事務連絡 *3 の中国産 GM コメの検査方法はその後改定を経て、最終的に 2007 年 2 月 20 日、厚生労働省医薬食品局食品安全部監視安全課長通知（以下通知とする）[1] として発出されたが、定性 PCR 法およびリアルタイム PCR 法を併用した定性試験である。

コメ加工品を含む GM 食品の検査は、各都道府県の衛生研究所および登録検査機関等で広く実施されているが、我々はこれらの検査機関における分析の信頼性の確認および向上を目的として、2001 年度より GM 食品検査に関する外部精度管理調査を試験的に実施している [2-5]。2007 年度は中国産 GM コメ混入事例の発生による社会的な影響を鑑み、中国産安全性未審査 GM コメの検査を対象とした外部精度管理調査の実施が求められた。

過去に実施した外部精度管理調査では、外部精度管理調査試料（調査試料）はいずれも GM 作物と宿主の非 GM 作物を混合する方法で調製したが、中国産安全性未審査 GM コメは入手不可能であったため、従来の方法では 2007 年度の調査試料を調製できなかった。一方、通知 [1] の検査方法は遺伝子組換えにより挿入された DNA 配列を検知の対象としており、内部精度管理用のポジティブコントロールとしてその DNA 配列を含むプラスミド DNA 溶液が市販されている。従って市販されているプラスミド DNA 溶液を用いた調査試料調製の可能性が考えられた。

本研究では、プラスミド DNA 溶液を用いて調製した調査試料を用いて 33 機関による外部精度管理調査を試験的に実施し、調査試料としての妥当性および、参加機関の中国産安全性未審査 GM コメ検査の信頼性について検討したので報告する。

Ⅱ　実験方法

1. 試薬、機器および測定方法

1) DNA 抽出

調査試料の調製に使用した非 GM コメ DNA 溶液は Genomic DNA Extraction Kit for Food Samples (Cartagen Molecular Science, Inc.) および付属のプロトコール APPENDIX A : Modified Protocol for Low DNA Samples により調製した。これ以外の DNA 抽出には GM quicker2 (㈱ニッポンジーン) を使用し、通知 [1] に従って操作した。なお、いずれの抽出においても、遠心分離には多用途小形遠心機 CF16RX（日立工機㈱）、恒温槽には DryThermoUnit DTU-2B（タイテック㈱）、吸光度測定には Gene Quant pro（GE ヘルスケアバイオサイエンス㈱）を使用した。

2) 定性 PCR

陽性対照用、CryIAc 検出用、Bt コメ検出用、Bt コメ確認用の各試験における PCR 増幅は、通知 [1] の各プライマー対および PCR 条件により実施した。なお、PCR 酵素には AmpliTaq Gold ™、PCR 装置には GeneAmp PCR System 9700（以上ライフテクノロジーズジャパン㈱）を使用した。増幅物の電気泳動は ultraPURE ™ Agarose-1000（ライフテクノロジーズジャパン㈱）、50×TAE（遺伝子工学研究用、㈱ニッポンジーン）により作製した 3% アガロースゲルを用い、泳動槽に Mupid（㈱ ADVANCE）を使用して実施した。また、エチジウムブロミド染色は前染色により行い、サイズマーカーには 20 bp DNA Ladder（タカラバイオ㈱）、画像解析にはプリントグラフ（アトー㈱）を使用した。

3) リアルタイム PCR

コメ陽性対照用試験、Bt コメ検出用試験（Duplex PCR）は通知 [1] の各プライマー対、プローブおよびリアルタイム PCR 条件により実施した。なお、マスターミックスには TaqMan Universal PCR Master Mix、リアルタイム PCR 装置には 7900HT Fast リアルタイム PCR システム 96 ウェル（以上ライフテクノロジーズジャパン㈱）を使用した。また、リアルタイム PCR の結果は Th. Line を 0.1 に設定してマルチコンポーネント解析（検出対象のプローブについて 50 サイクル時点での蛍光強度比 Rn を 10 サイクル時点の Rn で除した値を算出すること）を行い、マルチコンポーネント解析の値が 1.5 以上となった解析結果を陽性と判定した。

2. 外部精度管理調査試料の調製

1) 原料

検知対象の DNA 配列を含むプラスミドとして GM コメ Bt コメ陽性コントロールプラスミド（㈱ニッポンジーン、以下定性 PCR 用コントロールプラスミドとする）および高濃度 GM コメ Bt コメ最終確認検査用陽性コントロールプラスミド（市販の GM コメ Bt コメ最終確認検査用陽性コントロールプラスミドの高濃度品、㈱ニッポンジーンより供与、以下リアルタイム PCR 用コントロールプラスミドとする）を使用した。このほか神奈川県内で購入した国産上新粉を非 GM コメ DNA 溶液の抽出原料として、タイ産ビーフンを DNA 抽出操作の信頼性の確認を目的とした調査試料調製の原料として使用した。

*3　厚生労働省医薬食品局食品安全部監視安全課輸入食品安全対策室、事務連絡、モニタリング検査の実施について（中国産米加工品）、平成 18 年 9 月 26 日、(2006)。

*4　厚生労働省医薬食品局食品安全部監視安全課、プレスリリース、安全性未審査の中国産遺伝子組換え米の混入事例について、平成 19 年 1 月 26 日、(2007)。

2) コメ加工品粉砕物試料の調製および試験期間中の品質の確認

DNA抽出操作の確認を目的とした調査試料は、あらかじめ通知法[1]により中国産安全性未審査GMコメの混入がないことを確認したタイ産ビーフンを、孔径1.0 mmのスクリーンを装着した超遠心粉砕機ZM200（㈱レッチェ）で粉砕後、十分混合して作製した。これを25 mL容の遠沈管49本に2 gずつ分注し、コメ加工品粉砕物試料（陰性試料）とした。分注試料は使用まで-20℃で保存した。

分注試料から試料配布前および外部精度管理の試験期間終了後にそれぞれ6本を無作為に取り出し、DNAを抽出して定性PCR試験を実施し、検出結果を確認した。

3) 定性PCR用DNA溶液試料の調製および試験期間中の品質の確認

定性PCR用陽性試料は、検出下限の検討結果[*5]に基づいて、定性PCR用コントロールプラスミド（16800 copy/5 μL）を非GMコメDNA溶液で希釈し、定性PCR用コントロールプラスミドを容量比で1%および0.05%含む試料を調製した。このうち1%試料は陽性対照用、Cry1Ac検出用、Btコメ検出用、Btコメ確認用の全試験の検出を予定し、試料3（高濃度陽性試料）とした。また0.05%試料は陽性対照用、Cry1Ac検出用、Btコメ確認用の3試験の検出を予定し、試料1（低濃度陽性試料）とした。さらに遺伝子汚染の確認用として、非GMコメDNA溶液を試料2（陰性試料）として使用した。各試料はそれぞれ0.5 mL容のマイクロチューブ100本に40 μLずつ分注し、使用まで-20℃で凍結保存した。

試料1、試料2、試料3の分注試料は、試料配布前および外部精度管理調査の試験期間終了後にそれぞれ8本を無作為に取り出して定性PCR試験を実施し、調製した各試料が予定どおり検出できるか確認した。

4) リアルタイムPCR用DNA溶液試料の調製および試験期間中の品質の確認

リアルタイムPCR用陽性試料は、検出下限の検討結果[*5]に基づいて、リアルタイムPCR用コントロールプラスミド（250000 copy/5 μL）を非GMコメDNA溶液で希釈し、リアルタイムPCR用コントロールプラスミドを容量比で0.6%および0.12%含む試料を調製した。このうち0.6%試料はコメ陽性対照用試験および、Btコメ検出用試験（Duplex PCR）のGM63-TaqプローブおよびNGMr-Taqプローブのマルチコンポーネント解析の値が1.5以上の結果を予定し、試料A（高濃度陽性試料）とした。また0.12%試料はコメ陽性対照用および、Btコメ検出用試験（Duplex PCR）のNGMr-Taqプローブの解析の値が1.5以上の結果を予定し、試料B（低濃度陽性試料）とした。さらに遺伝子汚染の確認用として、非GMコメDNA溶液を試料C（陰性試料）として使用した。各試料はそれぞれ0.5 mL容のマイクロチューブ62本に40 μLずつ分注し、使用まで-20℃で凍結保存した。

試料A、試料B、試料Cの分注試料は、試料配布前および外部精度管理調査の試験期間終了後にそれぞれ8本を無作為に取り出して2試行でリアルタイムPCR試験を実施し、調製した各試料が予定どおり検出できるか確認した。

3. 外部精度管理調査試料の送付および参加機関における試験の実施

GM食品の検査を行っている検査機関のうち、事前に実施した調査で遺伝子組換え食品検査外部精度管理調査への参加を希望した機関数は33機関であった。これらの参加機関には、コメ加工品粉砕物試料1試料（1本）、定性PCR用DNA溶液試料3試料（試料1、試料2、試料3、各2本）、リアルタイムPCR用DNA溶液試料3試料（試料A、試料B、試料C、各1本）を実施要領および報告書書式とともに冷凍便で送付し、試料については到着後-20℃で保存するよう指示した。なお、実施要領には検査法として2007年2月20日付け通知[1]および2006年6月29日付けの通知[6]の方法を使用すること、および定性PCR用DNA溶液試料、リアルタイムPCR用DNA溶液試料測定の試行数および判定法の変更点を記載した。すなわち、定性PCR用DNA溶液試料においては、1試料につき2試行実施し、この結果を通知[1]におけるDNA抽出液2本それぞれ1試行の結果と読み換えて判定すること、およびリアルタイムPCR用DNA溶液試料は、コメ陽性対照用試験、Btコメ検出用試験（Duplex PCR）とも通知[1]の半分の試行数とし、1試料につき、コメ陽性対照用試験2ウェル（2試行）、Btコメ検出用試験4ウェル（1倍試料2試行＋2倍希釈試料2試行）を実施し、この結果から判定を行うよう指示した。

表1に通知[1]の実施手順と本外部精度管理調査における各試料の測定対象試験および試行数をまとめて示した。

Ⅲ 結果

1. 外部精度管理調査試料の調製

1) コメ加工品粉砕物試料の調製および分注済み試料の試験期間中の品質の確認

コメ加工品粉砕物試料（陰性試料）の分注試料を外部精度管理試験期間の前後に測定し、試験期間中の品質を確認した。その結果、試料配布前および試験期間終了後共に陽性対照用試験では全測定で予定長の増幅物が検出された。一方、Cry1Ac検出用試験、Btコメ検出用試験、Btコメ確認用試験では予定長の増幅物は検出されず、コメ加工品粉砕物試料は試験期間を通じて陰性の品質を保持していたことが明らかになった（表2）。

*5 井上ら、中国産安全性未審査遺伝子組換え米を対象とした外部精度管理調査における試料作製の検討、日本食品衛生学会第96回学術講演会講演要旨集、113（2008）。

表 1.　通知の実施手順および外部精度管理試料の測定対象試験

通知の実施手順の概略（上から順に実施）			外部精度管理試料の測定対象試験および試行数				判定
			コメ加工品粉砕物試料	定性 PCR 用 DNA 溶液試料		リアルタイム PCR 用 DNA 溶液試料	
試験名		試行数		陰性試料	陽性試料		
DNA 抽出		2	2				(260 nm/280 nm の吸光度比の目安は 1.7～2.0)
定性 PCR1	陽性対照用試験	2†	2†	2‡	2‡		陽性対照用試験の少なくとも 1 試行で 81 bp の増幅物を検出し、さらに Cry1Ac 検出用試験の少なくとも 1 試行で 90 bp の増幅物を検出した場合、定性 PCR2 に進む
	Cry1Ac 検出用試験	2†	2†	2‡	2‡		
定性 PCR2	Bt コメ検出用試験	2†			2‡		Bt コメ検出用試験で 147 bp、Bt コメ確認用試験で 120 bp のいずれかの増幅物を 1 試行でも検出した場合、リアルタイム PCR 試験に進む
	Bt コメ確認用試験	2†			2‡		
リアルタイム PCR	コメ陽性対照用試験	4††				2‡	コメ陽性対照用試験でマルチコンポーネント解析の値が 1.5 以上となる解析値が 1 つ以上あり、さらに Bt コメ検出用試験（Duplex PCR）の GM63-Taq プローブおよび NGMr-Taq プローブに対するマルチコンポーネント解析のいずれかで 1.5 以上となる解析値が 1 つ以上ある場合は陽性と判断する
	Bt コメ検出用試験（Duplex PCR）	8†††				4‡‡	

表の数字は各試験の試行数
†：2 抽出液× 1 試行　　††：2 抽出液× 2 試行
†††：2 抽出液×（1 倍抽出液 2 試行＋ 2 倍希釈抽出液 2 試行）
‡：1 試料につき 2 試行　　‡‡：1 倍試料 2 試行＋ 2 倍希釈試料 2 試行

表 2.　外部精度管理調査試料（コメ加工品粉砕物試料および定性 PCR 用 DNA 溶液試料）の定性 PCR による検出の確認結果

試験名	コメ加工品粉砕物試料 a)		定性 PCR 用 DNA 溶液試料 b)					
			試料 1		試料 2		試料 3	
	配布前	試験期間終了後	配布前	試験期間終了後	配布前	試験期間終了後	配布前	試験期間終了後
陽性対照用	6/6 c)	6/6	8/8	8/8	8/8	8/8	8/8	8/8
Cry1Ac 検出用	0/6	0/6	8/8	8/8	0/8	0/8	8/8	8/8
Bt コメ検出用	0/6	0/6	1/8	3/8	0/8	0/8	8/8	8/8
Bt コメ確認用	0/6	0/6	8/8	8/8	0/8	0/8	8/8	8/8
試料タイプ	陰性試料		陽性試料（低濃度）		陰性試料		陽性試料（高濃度）	

a) 6 容器の分注試料からそれぞれ 1 試行で抽出した DNA 溶液についてそれぞれの試験を 1 試行で実施
b) 8 容器の分注試料についてそれぞれの試験を 1 試行で実施
c) 予定長増幅物の検出数 / 測定数

2) 分注済み定性 PCR 用 DNA 溶液試料の試験期間中の品質の確認

定性 PCR 用 DNA 溶液試料の試料 1、試料 2、試料 3 の分注試料を試験期間の前後に測定し、試験期間中の品質を確認した。その結果、試料 1（低濃度陽性試料）では予定通り、陽性対照用試験、Cry1Ac 検出用試験、Bt コメ確認用試験の全測定で予定長の増幅物が検出された。試料 2（陰性試料）は、陽性対照用試験では全測定で予定長の増幅物が検出されたが、Cry1Ac 検出用試験、Bt コメ検出用試験、Bt コメ確認用試験では予定長の増幅物は全く検出されなかった。試料 3（高濃度陽性試料）では陽性対照用試験、Cry1Ac 検出用試験、Bt コメ検出用試験、Bt コメ確認用試験の全測定で予定長の増幅物が検出された（表 2）。この結果、定性 PCR 用 DNA 溶液試料はいずれも予定通り調製され、試験期間を通じて品質を維持していたことが確認された。

3) 分注済みリアルタイム PCR 用 DNA 溶液試料の試験期間中の品質の確認

リアルタイム PCR 用 DNA 溶液試料の試料 A、試料 B、試料 C の分注試料を試験期間の前後に測定し、試験期間中の品質を確認した。その結果、試料 A（高濃度陽性試料）では、コメ陽性対照用試験および Bt コメ検出用試験

(Duplex PCR) の GM63-Taq プローブおよび NGMr-Taq プローブのマルチコンポーネント解析の値は予定通り全て 1.5 以上だった。試料 B (低濃度陽性試料) では、コメ陽性対照用試験および Bt コメ検出用試験 (Duplex PCR) の NGMr-Taq プローブのマルチコンポーネント解析の値は予定通り全て 1.5 以上であった。試料 C (陰性試料) はコメ陽性対照用試験の全ウェルでマルチコンポーネント解析の値が 1.5 以上であったが、Bt コメ検出用試験 (Duplex PCR) では 1.5 以上となる解析はなかった (表 3)。この結果、リアルタイム PCR 用 DNA 溶液試料はいずれも予定通り調製され、試験期間を通じて品質を維持していたことが確認された。

2. 外部精度管理調査結果

1) コメ加工品粉砕物試料についての外部精度管理調査結果

コメ加工品粉砕物試料からの DNA 抽出では 260 nm/280 nm の吸光度比が精製度の目安である 1.7 ～ 2.0 の範囲外となった機関が 7 機関あった。しかし定性 PCR の陽性対照用試験の結果 (表 4) では全 33 機関が 2 試行共に予定長の増幅物を検出した。一方 Cry1Ac 検出用試験では 33 機関中 31 機関は予定長の増幅物を検出せず予定通り陰性と判定したが、2 機関では 2 試行とも予定長の増幅物を検出した。これら 2 機関はさらに Bt コメ検出用試験および Bt コメ確認用試験を行った。その結果、2 機関のうち 1 機関は両試験共に予定長の増幅物を検出せず、ここで陰性と判定した。残る 1 機関は両試験で 2 試行共に予定長の増幅物を検出し、定性 PCR の最終結果は陽性の判定となった。この結果、コメ加工品粉砕物試料は 33 機関中 32 機関が GM 陰性と判定し、正答率は 97% だった。なおデータは示していないが、定性 PCR 試験でコメ加工品粉砕物試料を陽性と判定した当該 1 機関は通知 [1] の手順に従ってリアルタイム PCR による最終確認試験を実施し、最終的に GM 陰性と判定した。

表 3. リアルタイム PCR 試験用外部精度管理試料のリアルタイム PCR による検出の確認結果

試験名	解析プローブ	試料 A		試料 B		試料 C	
		配布前	試験期間終了後	配布前	試験期間終了後	配布前	試験期間終了後
コメ陽性対照用試験		16/16 a)	16/16	16/16	16/16	16/16	16/16
Bt コメ検出用試験 (Duplex PCR)	GM63-Taq プローブ	16/16	16/16	13/16	16/16	0/16	0/16
	NGMr-Taq プローブ	16/16	16/16	16/16	16/16	0/16	0/16
試料タイプ		陽性試料 (高濃度)		陽性試料 (低濃度)		陰性試料	

各試料とも 8 容器の分注試料についてそれぞれの試験を 2 試行で実施
a) マルチコンポーネント解析の値が 1.5 以上となった解析数 / 測定数

表 4. 外部精度管理調査参加機関におけるコメ加工品試料および定性 PCR 用 DNA 溶液試料の定性 PCR 測定結果

試験名	コメ加工品粉砕物試料		定性 PCR 用 DNA 溶液試料					
			試料 1		試料 2		試料 3	
	陽性測定数	陽性機関数	陽性測定数	陽性機関数	陽性測定数	陽性機関数	陽性測定数	陽性機関数
陽性対照用	66/66	33/33	66/66	33/33	66/66	33/33	66/66	33/33
Cry1Ac 検出用	4/66	2/33	66/66	33/33	3/66	2/33	65/66	33/33
中間判定結果	2/33		33/33		2/33		33/33	
Bt コメ検出用	2/4	1/2	18/66	13/33	0/4	0/2	66/66	33/33
Bt コメ確認用	2/4	1/2	62/66	31/33	0/4	0/2	65/66	33/33
最終判定結果	1/33		33/33		0/33		33/33	
試料タイプ	陰性		陽性		陰性		陽性	
正答率 (%)	97		100		100		100	

陽性測定数: 予定長増幅物の検出数 / 総測定数
陽性機関数: 1 試行以上検出した機関数 / 総機関数
中間判定結果: 陽性対照用試験および Cry1Ac 検出用試験でともに 1 試行以上増幅物を検出した機関数 / 総機関数
最終判定結果: Bt コメ検出用試験および Bt コメ確認用試験を実施した機関のうちいずれかの試験で 1 試行以上増幅物を検出した機関数 / 総機関数

2) 定性 PCR 用 DNA 溶液試料についての外部精度管理調査結果

参加機関における定性 PCR 用 DNA 溶液試料の試験結果をまとめて表 4 に示した。陽性対照用試験では試料 1、試料 2、試料 3 共に全 33 機関が 2 試行とも予定長の増幅物を検出した。

試料 1 ではさらに Cry1Ac 検出用試験でも全 33 機関が 2 試行とも予定長の増幅物を検出した。試料 3 の Cry1Ac 検出用試験では 33 機関中 32 機関が 2 試行とも予定長の増幅物を検出したが、残る 1 機関は 2 試行のうち 1 試行のみの検出であった。しかし、この場合も判定は陽性となるため、試料 1、試料 3 については全 33 機関が予定通り Bt コメ検出用試験および Bt コメ確認用試験の実施に進んだ。試料 2 の Cry1Ac 検出用試験では 31 機関は 2 試行とも予定長の増幅物を検出せず GM 陰性と判定し、予定通りここで試験を終了した。しかし 2 機関は少なくとも 2 試行のいずれかで予定長の増幅物を検出し、陽性と判定したため、Bt コメ検出用試験および Bt コメ確認用試験の実施に進んだ。

Bt コメ検出用試験の結果、試料 1 で 1 試行でも予定長の増幅物を検出したのは 33 機関中 13 機関のみであった。一方、試料 3 では全 33 機関が 2 試行とも予定長の増幅物を検出し、全機関が陽性と判定した。また、試料 2 の測定を実施した 2 機関はいずれも予定長の増幅物を検出しなかった。

Bt コメ確認用試験の結果、試料 1 では 33 機関中 31 機関は 2 試行とも予定長の増幅物を検出したが、2 機関は 2 試行とも予定長の増幅物を検出しなかった。しかし、この 2 機関はいずれも Bt コメ検出用試験で予定長の増幅物を検出しているため、Bt コメ検出用試験の結果と合わせて試料 1 は予定通り全 33 機関が GM 陽性の最終判定となった。試料 3 の Bt コメ確認用試験においては 33 機関中 32 機関が 2 試行とも予定長の増幅物を検出した。残る 1 機関は 2 試行のうち 1 試行のみの検出であったが、試料 3 も予定通り全 33 機関が GM 陽性の最終判定となった。また、試料 2 の測定を実施した 2 機関は Bt コメ確認用試験でも予定長の増幅物を検出せず、最終的に陰性と判定し、試料 2 についても全機関が GM 陰性と最終判定した。以上の結果、定性 PCR 用 DNA 溶液試料の最終判定結果は正答率がいずれの試料も 100% であった。

また、参加機関における定性 PCR のブランク反応液の測定結果は全て陰性だったほか、定性 PCR 用陽性対照プラスミドの測定でも全機関が全試験で予定長の増幅物を検出し、これらの測定に問題は認められなかった。

3) リアルタイム PCR 用 DNA 溶液試料についての外部精度管理調査結果

参加機関におけるリアルタイム PCR 用 DNA 溶液試料の測定結果をまとめて表 5 に示した。コメ陽性対照用試験（1 倍液 2 試行）のマルチコンポーネント解析の値は試料 A、試料 B は全機関の全解析結果で、試料 C は 1 機関の 1 解析結果を除き全て 1.5 以上となり、全 33 機関で増幅が検出された。

Bt コメ検出用試験 4 ウェル（1 倍試料 2 試行＋ 2 倍希釈試料 2 試行）の GM63-Taq プローブ、NGMr-Taq プローブそれぞれのマルチコンポーネント解析結果は、試料 A（高濃度陽性試料）では全 33 機関で両プローブについて全て 1.5 以上となり、全機関が GM 陽性と判定した。試料 B（低濃度陽性試料）のマルチコンポーネント解析では、GM63-Taq プローブにおいて 27 機関では 4 ウェルとも 1.5 以上であったが、6 機関、1 倍試料で 3 解析、2 倍希釈試料で 4 解析の値が 1.5 未満であった。しかし NGMr-Taq プローブでは 33 機関の全解析で 1.5 以上となり、全機関が GM 陽性と判定した。

試料 C（陰性試料）のマルチコンポーネント解析では 33 機関中 28 機関が GM63-Taq プローブ、NGMr-Taq プローブ共に全て 1.5 を下回り、この時点で予定通り陰性と判定した。しかし 33 機関中 5 機関は、GM63-Taq プローブおよび NGMr-Taq プローブについての解析のうち少なくとも 1 つ以上 1.5 を上回る解析があった。これらの機関については報告書

表 5.　外部精度管理調査参加機関におけるリアルタイム PCR 用 DNA 溶液試料のリアルタイム PCR 測定結果

試験	解析プローブ	試料溶液の希釈倍	試料 A		試料 B		試料 C	
			陽性測定数	陽性機関数	陽性測定数	陽性機関数	陽性測定数	陽性機関数
コメ陽性対照用試験		× 1	66/66	33/33	66/66	33/33	65/66	33/33
Bt コメ検出用試験 (Duplex PCR)	GM63-Taq プローブ	× 1	66/66	33/33	63/66	33/33	0/66	0/33
		× 2	66/66		62/66		0/66	
	NGMr-Taq プローブ	× 1	66/66	33/33	66/66	33/33	1/66	1/33
		× 2	66/66		66/66		0/66	
最終判定結果			33/33		33/33		1/33	
試料タイプ			陽性		陽性		陰性	
正答率 (%)			100		100		97	

陽性測定数：マルチコンポーネント解析の値が 1.5 以上となった解析数 / 総測定数
陽性機関数：マルチコンポーネント解析の値が 1.5 以上となった解析がある機関数 / 総機関数
最終判定結果：コメ陽性対照用試験においてマルチコンポーネント解析に 1.5 以上の解析値があり、かつ Bt コメ検出用試験 (Duplex PCR) のいずれかのプローブについての解析で 1.5 以上となる解析値があった機関数 / 総機関数

とともに提出をうけたGM63-TaqプローブおよびNGMr-Taqプローブの Amplification plot の出力図を確認した。その結果、5機関のうち1機関ではNGMr-Taqプローブの1解析で指数関数的な増幅が確認でき、報告通り陽性の結果であることが確認された。残る4機関はいずれかのプローブで1.5以上となる解析結果があるにもかかわらず陰性と報告しているが、Amplification plot の出力図で増幅が認められた測定はなく、陰性の判定で間違いないことが確認された。なお、これら4機関はいずれもリアルタイムPCR装置にABI7700を使用していることが判明した。

以上の結果、陽性試料の試料A、試料Bは予定通り全33機関がGM陽性と判定し、正答率はいずれも100%だった。しかし陰性試料の試料Cは1機関が誤ってGM陽性と判定したため正答率は97%となった。なお、リアルタイムPCR試験におけるコメ陽性対照用試験、Btコメ検出用試験（Duplex PCR）の試行数はいずれも通知[1]の半分の設定であったが、陽性試料を陰性試料と判定した機関はなく、今回の外部精度管理調査の結果に影響はないと考えられた。

リアルタイムPCR測定における陽性対照プラスミドの測定結果には特に問題は認められなかったが、ブランク反応液の測定では、コメ陽性対照用試験においてマルチコンポーネント解析の値が1.5以上となり、Amplification plotにおいても増幅が確認された機関が4機関あった。

Ⅳ 考察

外部精度管理調査におけるコメ粉砕物試料からのDNA抽出では260 nm/280 nmの吸光度比が1.7～2.0の範囲外となった機関もあったが、抽出DNAを鋳型とした定性PCRの陽性対照用試験では全抽出で増幅物が検出されたことから、DNAの精製度は妥当であったと考えられた。なお陰性試料にもかかわらずCry1Ac検出用試験では、33機関のうち2機関から予定長の増幅物が検出されたとの報告があったが、Cry1Ac検出の原因は後述する定性PCR操作または電気泳動操作にあると考えられた。

定性PCR用DNA溶液試料のうち陽性試料の分析では、試料1（低濃度陽性試料）、試料3（高濃度陽性試料）ともにすべての参加機関がGM陽性と判定し、定性PCRの検出感度に問題のある機関はないものと考えられた。一方、試料2（陰性試料）の分析では、Cry1Ac検出用試験で予定長の増幅物を検出した機関が2機関あった。このうち1機関はコメ加工品粉砕物試料のCry1Ac検出用試験で増幅物を検出した機関と同一であり、DNA抽出、PCR操作、電気泳動の操作区域、およびDNA抽出、PCR操作のピペットが共通との報告があることから、PCR操作の際に遺伝子混入を起こした可能性が高いと考えられた。また、Cry1Ac検出用試験で増幅物を検出したもう1機関では、他機関と比べてCry1Ac検出用試験およびBtコメ検出用試験で非特異的増幅物が多く検出されていることから、PCRの際に遺伝子混入があったものと考えられた。このほか、定性PCRではコメ加工品粉砕物試料のCry1Ac検出用試験で増幅物を検出した機関も含め、電気泳動操作に改善の余地がある機関が複数見受けられた。今回の定性PCRでは、予定長の増幅物が陽性対照用試験では81 bp、Cry1Ac検出用試験では90 bp、Btコメ検出用試験では147 bp、Btコメ確認用試験では120 bpであるにもかかわらず100 bp Ladderのサイズマーカーを使用した機関が多く、またアガロース濃度が2%のゲルを使用した機関の泳動像は増幅物の分離が不十分な結果が多かった。このため、非特異的増幅物が認められた場合、増幅物のサイズの確認が難しく、予定長の増幅物の有無を判定することが困難な場合があった。なお、通知[1, 6]にはアガロースゲル濃度は増幅産物のバンド長に合わせて決めること、および増幅物をサイズマーカーと比較して判定することは記載されているが、いずれについても選択の目安や具体的指示は記述されていない。本外部精度管理調査の結果、サイズマーカー、ゲル濃度の適正な選択が、定性PCR操作の信頼性の向上に欠かせないことが明らかになったことから、定性PCRを使用する検知法には、サイズマーカーおよびゲル濃度についての具体例を示すことが重要であると考えられた。

リアルタイムPCR用DNA溶液試料のうち陽性試料の試料A（高濃度陽性試料）および試料B（低濃度陽性試料）のBtコメ検出用試験（Duplex PCR）におけるNGMr-Taqプローブ、GM63-Taqプローブを対象としたマルチコンポーネント解析ではともに全機関がGM陽性と判定し、参加機関のリアルタイムPCRの測定感度には問題がないものと考えられた。一方、試料C（陰性試料）ではBtコメ検出用試験（Duplex PCR）のNGMr-Taqプローブによる解析で1機関の1解析が1.5以上となった。この機関は定性PCR用DNA溶液試料の試料2、およびコメ加工品粉砕物試料のCry1Ac検出用試験で増幅物を検出した機関とは別機関であるが、ピペットまたは操作区域の共用により、陽性プラスミドあるいはPCR増幅産物の混入があったものと考えられた。

一方、試料CのBtコメ検出用試験（Duplex PCR）でマルチコンポーネント解析の値が1.5以上となる解析があったもののAmplification plotで増幅が確認されなかった4機関は、いずれもリアルタイムPCR装置にABI7700を使用していた。これらの機関では、ブランク溶液のマルチコンポーネント解析結果もNGMr-Taqプローブ、GM63-Taqプローブのいずれかまたは両方で1.5以上となっていた（表6）。またそのAmplification plotでは、蛍光強度のベースラインがサイクル数に比例して徐々に上昇しているのが観察された。したがってこれらの機関では使用装置に依存したベースラインの上昇により、マルチコンポーネント解析の値が1.5以上となったものと考えられた。なお、ABI7700を使用した機関でも、ブランク溶液の測定で、マルチコンポーネント解析の結果が全て1.5未満の機関では、試料Cで1.5を超えた機関はなかった。以上の結果から、一般にABI7700は他の測定機に比べて蛍光強度のベースラインがサイクル数に比例して上昇しやすいため、マルチコンポーネント解析の値が大きく計算される場合があることが示唆され、リアルタイムPCRを用いた定性試験でのAmplification plot確認の重要性が示された。