遺伝子組換え(耐熱性α-アミラーゼ産生)トウモロコシ3272に適したDNA抽出法の検討および系統特異的定量検知法の開発と性能評価

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Original

Selection of Suitable DNA Extraction Methods for Genetically Modified Maize 3272, and Development and Evaluation of an Event-Specific Quantitative PCR Method for 3272

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A novel real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) maize, 3272. We first attempted to obtain genome DNA from this maize using a DNeasy Plant Maxi kit and a DNeasy Plant Mini kit, which have been widely utilized in our previous studies, but DNA extraction yields from 3272 were markedly lower than those from non-GM maize seeds. However, lowering of DNA extraction yields was not observed with GM quicker or Genomic-tip 20/G. We chose GM quicker for evaluation of the quantitative method. We prepared a standard plasmid for 3272 quantification. The conversion factor (Cf), which is required to calculate the amount of a genetically modified organism (GMO), was experimentally determined for two real-time PCR instruments, the Applied Biosystems 7900HT (the ABI 7900) and the Applied Biosystems 7500 (the ABI7500). The determined Cf values were 0.60 and 0.59 for the ABI 7900 and the ABI 7500, respectively. To evaluate the developed method, a blind test was conducted as part of an interlaboratory study. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation (RSDR). The determined values were similar to those in our previous validation studies. The limit of quantitation for the method was estimated to be 0.5% or less, and we concluded that the developed method would be suitable and practical for detection and quantification of 3272.

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Key words: 3272; event-specific; genetically modified (GM); real-time PCR; DNA extraction method; maize

Introduction

Genetically modified (GM) crops are increasingly being developed worldwide, with the global area of GM crops undergoing a continuous increase from 1.7 million hectares in 1996 to 181 million hectares in 2014[1]. In Japan, to provide consumers with accurate information about food ingredients, a labeling system has been legislated for authorized GM crops or their derived foods. The Japanese GMO labeling system had been defined by two laws, the "JAS law" and the "Food Sanitation law", and is now defined by the newly legislated "Food Labeling Act (Law number: Act No. 70 of 2013). The thresholds for approved GM maize and soybean events have both been set at 5%[2),*1].

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Traditionally, maize (*Zea mays* L.) has mainly been consumed as food or feed, and thus the initially developed GM maize events were focused primarily on herbicide tolerance and/or insect resistance. Recently, however, maize has also been increasingly used for the production of ethanol as biofuel. 3272 is a GM maize that includes a synthetic gene encoding a thermostable α-amylase (AMY797E) derived from *Thermococcus* species bacteria[3], and this GM maize event was developed to improve the efficiency of ethanol production. The thermostable α-amylase is capable of hydrolyzing starch at high temperature, which is the first step in producing ethanol, and this results in a significant improvement in

*1 Notification No. 139 (Mar. 30, 2015); Consumer Affairs Agency, Government of Japan (2015) http://www.caa.go.jp/foods/pdf/150330_tuchi-bun.pdf

ethanol production[4]. 3272 is expected to be utilized mainly for fuel ethanol production as originally intended. However, the possibility of unintentional comingling of 3272 in food or feed cannot be ruled out. The commercial utilization of 3272 as food in Japan was approved in 2010*2, in the case of unintentional comingling of 3272 with other food or feed. In order to detect and quantify approved GM maize, construct-specific quantitative methods for five GM maize events (Bt11, Bt176, GA21, MON810, and T25) and a screening quantitative method targeting Cauliflower Mosaic Virus 35S promoter (P35S) and GA21 were adopted as Japanese standard analytical methods*3. However, because the P35S region is not introduced in 3272, an event-specific quantitative detection method for 3272 is required.

In this study, we developed a new real-time PCR-based event-specific quantitative method for 3272 and then validated it with an interlaboratory study. During the course of the development, it was found that only a small amount of DNA was extracted from 3272 maize seeds using certain DNA extraction kits. We therefore selected suitable DNA extraction kits for the 3272 event.

Materials and Methods

Plant materials

The soybean and maize seeds, 3272, Bt11, Event176, GA21, MIR162, and MIR604 were kindly provided by Syngenta Seeds AG (Basel, Switzerland). MON810, MON863, MON88017, NK603, MON89788, and RRS were kindly provided by Monsanto Company (St. Louis, MO, USA). TC1507 and DAS59122 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). A2704–12 was kindly provided by its developer and T25 was directly imported from the USA. QC9651 maize from Quality Technology International, Inc., (Elgin, IL, USA) was used as a non-GM maize. Seeds of the domestically harvested rice (*Oryza sativa*) variety Kinuhikari, wheat (*Triticum aestivum*) variety Haruyutaka, and barley (*Hordeum vulgare*) variety Harrington were purchased at a market in Japan.

DNA extraction

Maize genomic DNA was extracted with a DNeasy Plant Maxi kit (Maxi kit) (Qiagen, Hilden, Germany), and Genomic-tip 20/G (Qiagen) according to the JAS analytical test handbook*4, and with a DNeasy Plant Mini kit (Mini kit) (Qiagen) and GM quicker (NIPPON GENE, Tokyo, Japan) according to the Japanese standard analytical methods*3. Each DNA extraction was performed 12 times. Approximately 1.0 g of the ground samples was used for the Maxi kit, GM quicker, and Genomic-tip 20/G, and the DNA was finally dissolved in 50 μL of 10 mM Tris–HCl (pH 8.0), 1 mM EDTA buffer (TE). For the Mini kit, around 2.0 g of the sample was used and the DNA was finally dissolved in 50 μL of TE.

The concentration and quality of the extracted DNA solutions were evaluated by measuring ultraviolet (UV) absorbance with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The concentrations of genomic DNA solutions were adjusted to 10 and 20 ng/μL for conventional and real-time PCR analyses, respectively.

Oligonucleotide primers and probes

For the specific detection of 3272, a pair of primers (5′-TCATCAGACCAGATTCTCTTTTATGG-3′ and 5′-CGTTTCCGCCTTCAGTTTA-3′) and a fluorescent dye-labeled probe (5′-ACTGCTGACGCGGCCAAACACTG-3′) were used. The sequences of these primers and probe were taken from a report by the Institute for Health and Consumer Protection, the European Commission's Joint Research Centre (IHCP-JRC) (Ispra, Italy)*5. As a maize-specific endogenous reference, maize *starch synthase IIb* (*SSIIb*) was used for quantitative analysis. For the specific detection of *SSIIb*, the primers (5′-CCAATCCTTTGACATCTGCTCC-3′ and 5′-GATCAGCTTTGGGTCCGGA-3′) and fluorescent dye-labeled probe (5′-AGCAAAGTCAGAGCGCTGCAATGCA-3′) were used[5]. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC Co., Ltd. (Kanagawa, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ ends and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ ends.

Preparation of calibrant plasmid

Target sequence fragments from 3272 and the endogenous maize *SSIIb* gene were synthesized as a single oligonucleotide in tandem and inserted into a pUC19-derived vector. The constructed plasmid, designated as p3272, was purified by equilibrium centrifugation in a CsCl gradient. The concentration of the linearized DNA was calculated from the UV absorbance, measured with a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA), and converted to molar concentration. The DNA solution was then diluted to theoretical concentrations of 20, 125, 1,500, 20,000, and 250,000 copies per 2.5 μL, with 5 ng/μL of ColE1 DNA (NIPPON GENE) solution.

*2 List of products that have undergone safety assessment and been announced in the Official Gazette (Jan 15, 2015) of the Department of Food Safety, Ministry of Health, Labour, and Welfare; http://www.mhlw.go.jp/english/topics/food/pdf/sec01-2.pdf

*3 Notification No. 201 (Nov. 16, 2012); Consumer Affairs Agency, Government of Japan (2012) http://www.caa.go.jp/foods/pdf/syokuhin960.pdf

*4 Japanese Agricultural Standard (JAS) analytical test handbook: Genetically modified food quality, labeling analysis manual for individual products (2012). The Food and Agricultural Material Inspection Center, Japan. http://www.famic.go.jp/technical_information/jashandbook/index.html

*5 Event-specific method for the quantification of maize 3272 using real-time PCR; http://gmo-crl.jrc.ec.europa.eu/summaries/3272_validated_Method.pdf

Preparation of test samples

To evaluate the quantitative method, we used five mixing levels of test materials containing 0, 0.50, 1.0, 5.0, and 10.0% of 3272. To prepare the mixed samples, the GM and non-GM seeds were separately ground, and then mixed on a weight-to-weight basis as described previously[6, 7]. Genomic DNA was extracted from the ground materials using a GM quicker (NIPPON GENE). The concentration and quality of extracted DNA solutions were evaluated by measuring the UV absorbance with an ND-1000 spectrophotometer. The maize genomic DNA solutions were adjusted to concentrations of 10 and 20 ng/μL for conventional and real-time PCR analyses, respectively.

Conventional PCR analysis

PCR amplifications were carried out as described previously[8]. Twenty-five nanogram of template DNA, 200 μM deoxyribonucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.625 U of AmpliTaq Gold polymerase (Life Technologies) and 0.5 μM each primer were added to a 25 μL reaction volume. The thermal cycling conditions using a GeneAmp PCR system 9700 thermal cycler (Life Technologies) were set as 10 min at 95℃ and 40 cycles of 30 sec at 95℃, 30 sec at 60℃ and 30 sec at 72℃, followed by a final extension at 72℃ for 7 min. Five microliters of PCR products were electrophoresed on 3.0% agarose gel supplemented with 0.5 μg/mL of ethidium bromide in Tris–acetate–EDTA (TAE) buffer.

Real-time PCR analysis

TaqMan® real-time PCR assays were carried out using the Applied Biosystems 7900HT (the ABI 7900) or the Applied Biosystems 7500 (the ABI 7500) (Life Technologies) in 25 μL final volume reactions consisting of 50 ng of sample DNA, 12.5 μL Universal Master Mix (Life Technologies), 0.5 μM each primer pair, and 0.2 μM probe. The step-cycle program was as follows: 2 min at 50℃, 10 min at 95℃, 45 cycles, 30 sec at 95℃, and 1 min at 59℃. To quantify the DNA copy numbers, calibration curves that were created using 20, 125, 1,500, 20,000, and 250,000 copies of p3272 were used. In the reaction plate, each sample was measured in triplicate.

Interlaboratory study

The interlaboratory study was performed with the ABI 7900 and the ABI 7500 independently and consisted of 2 separate stages, *i.e.*, measurement of the Cf value and a blind test. All measurements were conducted by 10 laboratories for the ABI 7900 and 3 laboratories for the ABI 7500. Experimental protocols were provided by the National Food Research Institute (NFRI). Quantitative real-time PCR was performed with NFRI-provided primers, probes, the Universal Master Mix, and the blind DNA solutions.

The first stage was the experimental determination of the Cf value as the ratio of the copy number of recombinant DNA (r-DNA) to the taxon-specific sequence in the GM plant genome. To calculate the Cf value for 3272, we extracted the genomic DNA from genuine GM seeds and determined the copy numbers of r-DNA and taxon-specific sequences. The measurement was conducted twice at each laboratory. The Cf value for each real-time PCR instrument was separately determined as the mean of the obtained values.

A blind test was carried out as the second stage. Blind samples designed as blind duplicates of the maize genomic DNAs extracted from 5 different concentrations of 3272, *i.e.*, 0, 0.50, 1.0, 5.0, and 10.0%, were sent to the participants. All participants were requested to submit the data from the real-time PCR analyses. All submitted data were analyzed by the Cochran's test[9] and by the Grubbs' test[10, 11].

Results and Discussion

Selection of a suitable DNA extraction kit for 3272

In our previous studies, we used a Maxi kit when extracting DNA from many kinds of GM maize and soybean events, particularly in the context of interlaboratory studies. In the present work, therefore, we first attempted to extract DNA from 3272 using a Maxi kit, and were surprised to find that only a small amount of DNA was extracted from 3272 seeds compared to non-GM maize (Table 1). The yield of DNA from 3272 was around 40.0 ng/μL, which corresponded to one-tenth of that obtained from non-GM maize. Therefore, we next tried to use the other DNA extraction kits listed in the Japanese standard method, namely, Mini kit*3, GM quicker*3, and Genomic-tip 20/G*4. Maize genomic DNA was extracted from 3272 and non-GM, and the yields were compared (Table 1). Student's *t*-test indicated that, when using the Maxi kit and Mini kit, the yields from 3272 were significantly lower than those obtained from non-GM. When using GM quicker and Genomic-tip 20/G, however, no significant difference in yield was observed between the 3272 and non-GM maize. These results sug-

Table 1. Comparison of DNA yield (ng/μL) of non-GM and 3272 maize using four DNA extraction methods

	Non-GM				3272				*p*-Value (comparison with non-GM)
	Mean (ng/μL)	SD	260/280	SD	Mean (ng/μL)	SD	260/280	SD	
Maxi kit	333.34	98.14	1.5982	0.0977	39.98	31.62	4.2297	5.8166	7.5×10^{-8}
Mini kit	121.90	17.24	1.8188	0.0115	85.88	12.11	1.7798	0.0230	5.7×10^{-6}
GM quicker	79.90	5.14	1.9520	0.0236	80.99	8.48	1.9397	0.0115	0.35
Genomic tip 20/G	1332.44	179.08	1.8534	0.0054	1308.21	135.76	1.8047	0.0222	0.36

Table 2. Comparison of DNA yield (ng/μL) from maize samples containing five different concentrations of 3272

	0%	1%	*p*-Value (comparison with 0%)	5%	*p*-Value (comparison with 0%)	10%	*p*-Value (comparison with 0%)	50%	*p*-Value (comparison with 0%)	100%	*p*-Value (comparison with 0%)
Mean	259.83	214.34	0.18	152.35	7.6×10^{-4}	108.18	1.1×10^{-4}	57.77	1.6×10^{-6}	35.99	8.0×10^{-7}
SD	45.35	106.58		40.73		48.34		28.28		45.35	
RSD	17.45	49.73		26.73		44.68		48.96		64.11	

SD; Standard deviation
RSD; Relative standard deviation

Table 3. Quantified copy numbers of the 3272 and SSIIb PCR target region in 50 ng of template DNA from maize samples containing four different concentrations in Table 2

		1%	5%	10%	50%
3372	Mean	176.63	874.06	1854.26	8613.52
	SD	44.50	41.52	250.01	902.15
SSIIb	Mean	25989.77	25555.24	26282.04	27064.36
	SD	1061.77	1457.42	3070.77	1888.44
Copy number ratio of 3272/*SSIIb*		6.80×10^{-3}	3.42×10^{-2}	7.06×10^{-2}	3.18×10^{-1}
Ratio of 3272/SSIIb to 1% sample[a]		1.00	5.03	10.38	46.83

SD; Standard deviation
[a]Copy number ratio of each % sample/copy number ratio of 1% sample

gested that the DNA contents of 3272 seeds were not different from those of non-GM seeds, but the DNA extraction yields from 3272 seeds using a Maxi kit or Mini kit were remarkably low. The Maxi kit and Mini kit are both silica-membrane-based DNA purification systems, and an anion-exchange column is used in place of a silica-membrane in Genomic-tip 20/G. However, GM quicker is also a silica-membrane-based DNA purification system. The reason for the low DNA extraction yields with the Maxi kit and Mini kit is not clear at present.

We then evaluated the effects of different amounts of 3272 on the DNA extraction yield with a Maxi kit; for this purpose we used 6 mixing levels of maize ground samples containing 0, 1, 5, 10, 50, and 100% of 3272. The results showed that the DNA extraction yields were significantly reduced in the case of maize samples containing 5% or more, but not 1%, of 3272 (Table 2).

Subsequently, when using the Maxi kit-extracted DNA solution, the copy numbers of the PCR target regions of 3272 and *SSIIb*, except in the case of the samples consisting of 100% 3272, were quantified with the ABI 7900 using a calibrant standard plasmid, p3272 (Table 3). For the 100% 3272 samples, the concentrations of several extracted DNA solutions were less than 20 ng/μL, and were not sufficient to prepare 50 ng of template DNA for the real-time PCR analysis. Although the DNA extraction yield itself was significantly reduced, the copy number ratios of 3272/*SSIIb* seemed to be not so much different from the concentration of 3272 in each mixing sample, suggesting that the DNA extraction efficiencies from 3272 and non-GM maize seeds in these mixing samples were not substantially different. Thus, the results suggested that some constituent(s) derived from 3272 seeds may have interfered with DNA extraction or purification when the Maxi kit or Mini kit was used.

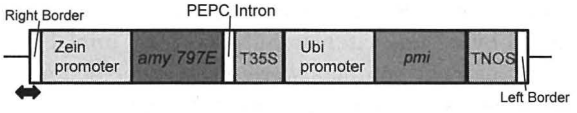

Fig. 1. A schematic diagram of the target position in 3272

The event-specific target sequence is at the 5'-flanking region between the exogenous insert and host maize DNA.

We decided to use the GM quicker for DNA extraction from 3272 seeds in the following evaluations because, first of all, lowering of DNA extraction yields was not observed with GM quicker and Genomic-tip 20/G (Table 1), so, the biases between the mixing levels and quantification results of 3272 would be expected to be small compared to those with the Maxi kit and Min kit. Also, GM quicker is rapid and easy-to-use, compared to Genomic-tip 20/G, and is an official Japanese standard method for GM quantification analyses*[3].

The PCR specificity for 3272

For specific detection of 3272, an event-specific segment was amplified at the 5′ flanking region between the native maize genomic DNA and r-DNA (Fig. 1). The specificity of the primer set was first confirmed by conventional PCR analysis. The expected 95-bp product was detected using genomic DNA solutions only from 3272, but not from non-GM maize, other GM maize events, GM soybean events, rice, wheat, and barley, or the no-template control (Fig. 2 A). The specificity of the primer and probe was also confirmed by real-time PCR analysis. The expected amplification curves were observed using a template of 3272 genomic DNA, but were not observed

for the other samples (Fig. 2 B).

Determination of the Cf value for 3272

The Cf value is required for converting the copy number ratio of recombinant DNA to taxon-specific DNA into weight-based GMO content. To determine the experimental Cf value for 3272, we measured the copy numbers of *SSIIb* and 3272 in the DNA extracted from 3272 seeds. The Cf value was determined with two real-time PCR instruments (the ABI 7900 and the ABI 7500) independently, from the results of 10 laboratories for the ABI 7900 and 3 laboratories for the ABI 7500; all measurements were repeated twice. All the submitted data were subjected to Cochran's test ($p<0.025$) and Grubb's test ($p<0.025$) to remove outlier laboratories with an extreme variation and an extreme average level, respectively, according to the harmonized guidelines of AOAC[12], and no laboratory was eliminated. The determined Cf values as the mean of values with the ABI 7900 and the ABI 7500 were 0.60 and 0.59, respectively (Table 4).

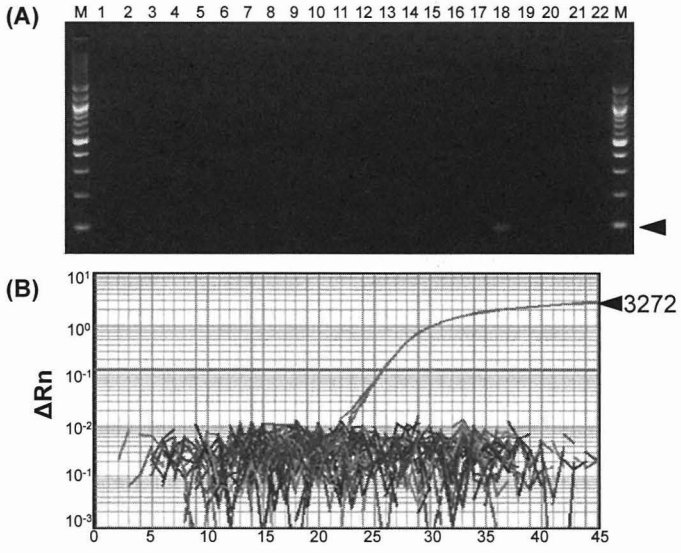

Fig. 2. Specificity test for the 3272-specific PCR

(A) Conventional PCR analysis. This agarose gel (3.0%) electrophoretogram of the amplified PCR product corresponds to the 95 bp sequence of 3272. The arrowhead indicates the expected amplified product. Lanes 1–11 showed the results for 11 GM maize events, namely, NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MIR604, MON88017, DAS59122, and MON863, respectively; lanes 12 and 13, non-GM maize and non-GM soy, respectively; lanes 14–16, three GM soybean events, namely, RRS, MON89788, and A2704–12, respectively; lanes 17 and 18, GM maize events, MIR162 and 3272, respectively; lanes 19–22, rice, wheat, barley, and no template, respectively. Lane M shows 100 bp ladder size markers. (B) Real-time PCR analysis. The same set of plant genomic DNAs described in (A) were used as templates. The real-time PCR analysis was performed in one reaction plate, and the resultant amplification plot is shown. This analysis was performed with 7900HT. Only the 3272 genomic DNA gave ideal PCR amplification.

Evaluation of the PCR quantification by an interlaboratory study

We conducted an interlaboratory evaluation of the developed quantitative method as a blind test performed by 10 laboratories using the ABI 7900. All the participants received primers, probes and blind samples as DNA solutions consisting of five different concentrations of 3272, and the measurement was performed twice. The blank sample with 0% of 3272 was used to determine outlier laboratories, and no laboratory was eliminated. Cochran's test and Grubbs' test were conducted on all submitted data, and one Cochran outlier was detected in the 1.0% sample. The trueness and precision were determined as previously described[5)–7)]. The mean, bias (mean-value, %), RSDr, and RSD$_R$ of blind samples were measured (Table 5). The determined bias, RSDr, and RSD$_R$ for the ABI 7900 ranged from −2.9 to 9.2%, from 10.8 to 13.6%, and from 11.3% to 26.4%, respectively. The standard curve was extrapolated for data below 20

Table 4. Empirically obtained conversion factor for 3272

7900			7500		
Mean	SD	RSD	Mean	SD	RSD
0.601	0.045	7.49	0.590	0.018	3.05

SD; Standard deviation
RSD; Relative standard deviation

Table 5. Summary of accuracy and precision statistics for real-time PCR by ABI PRISM 7900HT

% (w/w)	Retained labs	Trueness		Precision		Detection limit
		Means	Bias	RSDr[a], %	RSD$_R$[b], %	
		GMO amount, %	True value, %			Below 20 copies[c]
0.50	10	0.55	9.2	12.9	26.4	0/20
1.0	9	1.07	6.6	11.5	17.5	0/18
5.0	10	5.35	7.1	10.8	11.3	0/20
10.0	10	9.72	−2.9	13.6	16.0	0/20

[a]RSDr; Repeatability relative standard deviation
[b]RSD$_R$; Reproducibility relative standard deviation
[c]Below 20 copies are expressed as the ratio of the number of retained data below 20 copies/the total number of retained data.

copies in our methods because there was no calibrant below 20 copies. In Table 5, all the measured values of 0.50% samples were over 20 copies. In Europe, the acceptance criteria for validation of analytical methods are defined by the European Network of GMO Laboratories (ENGL)*[6]. The limit of quantification (LOQ) is defined as the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy with an RSDr $\leq$ 25%. In the criteria, it is also defined that RSDR should be less than 35%. All obtained RSDr and RSDR were below the acceptable level. Moreover, the RSDr and RSDr here were similar to, or within a narrower range than, those previously reported for GMO events[13]. Therefore, we estimate that the LOQ for 3272 was around 0.50% by this method.

In conclusion, we developed a specific quantification method for GM maize 3272. Surprisingly, maize genome DNA was hardly extracted from 3272 using a Maxi kit or Mini kit, which are listed in the Japanese standard methods. We found that, if a considerable amount of 3272 commingling is expected in maize samples, GM quicker is the most suitable DNA extraction method for quantification.

The Cf value was experimentally determined. The LOQ, trueness, and precision of this method were equivalent to or better than those of previous methods[5)–7), 13)]. Therefore, we concluded that the developed method would be applicable for the detection and quantification of 3272 to monitor the validity of the food labeling system in Japan.

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Japan Grain Inspection Association, Tokyo, Japan

Life Technologies Japan, Tokyo, Japan

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遺伝子組換え（耐熱性α-アミラーゼ産生）トウモロコシ3272に適したDNA抽出法の検討および系統特異的定量検知法の開発と性能評価（報文，英文）

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遺伝子組換えトウモロコシ3272系統のリアルタイムPCRを用いた系統特異的定量分析法を開発した．DNA抽出に関しては，DNeasy Plant Maxi kitおよびDNeasy Plant Mini kitを用いたところ，非組換え種子に比べて抽出DNA量が有意に低かったが，GM quickerおよびGenomic-tip 20/Gを用いたところ，このような差は見られなかった．GM quickerで抽出精製したDNAを用いて試験室間共同試験を実施し，混入率算出の際に必要となる係数である内標比を決定した．さらに，ブラインド定量試験を実施したところ，本定量分析法の定量下限値は0.5%程度と見積もられた．以上の結果から，本分析法が，実際の検査に適用可能であることが示された．

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