level for each GM event were divided into 100 sample tubes. Ten DNA samples were randomly selected from the 100 tubes prepared and quantitative PCR was performed twice using specific PCR systems. The calculated copy numbers were then converted into GMO amounts (%) on a weight basis. Homogeneity was evaluated according to a protocol for proficiency testing (Food Analysis Performance Assessment Scheme, 2002) based on the study by Fearn and Thompson (2001). Statistical analysis indicated that all test materials were homogeneous and met the requirements for the interlaboratory study.

Interlaboratory evaluation The interlaboratory study was carried out as a blind test using the ABI PRISM 7700 and 7900HT, as described previously (Kodama *et al.*, 2009). Maize genomic DNAs were extracted from the 6 different mix ratios for each GM event: 0, 0.25, 0.50, 1.0, 5.0 and 10.0%. Blind samples were combinations of 5 different concentrations of DNA solutions, which were carefully designed and allotted from the 6 concentrations mentioned above. Experimental protocols were provided by National Food Research Institute (NFRI). Quantitative real-time PCR was performed with primers, probes, Universal Master Mix, and the blind DNA solutions supplied by NFRI. All submitted data were analyzed by Cochran's test in order to remove laboratories with extreme variations and by Grubb's test in order to remove any laboratories with extreme average levels, as described in the guidelines (AOAC, 2002).

Results and Discussion

Specificity of PCR systems designed for MON863, NK603, TC1507 and T25 We selected event-specific segments for MON863, NK603 and T25, and a construct-specific segment for TC1507 (Fig. 1). Event-specific segments were designed to amplify the junction sites between the native maize genomic DNA and the r-DNA of each GM event. For T25, we previously designed and used a construct-specific segment that targets the junction between the *pat* gene and the CaMV terminator (Kodama *et al.*, 2009; Kuribara *et al.*, 2002; Shindo *et al.*, 2002). However, as shown in Figs. 1 C and D, during the course of developing a method for TC1507, the segment was also found to be present in the TC1507 construct, meaning that the region could no longer be used for T25 specific detection and quantification. Therefore, we designed a new event-specific segment for T25. The specificities of these designed primers were confirmed by qualitative PCR. No unexpected PCR products were detected using genomic DNAs from non-GM maize, the 8 lines of GM maize, non-GM soybean, RRS, and rice, and wheat and barley (Fig. 2).

Construction of calibrant plasmid In our laboratory,

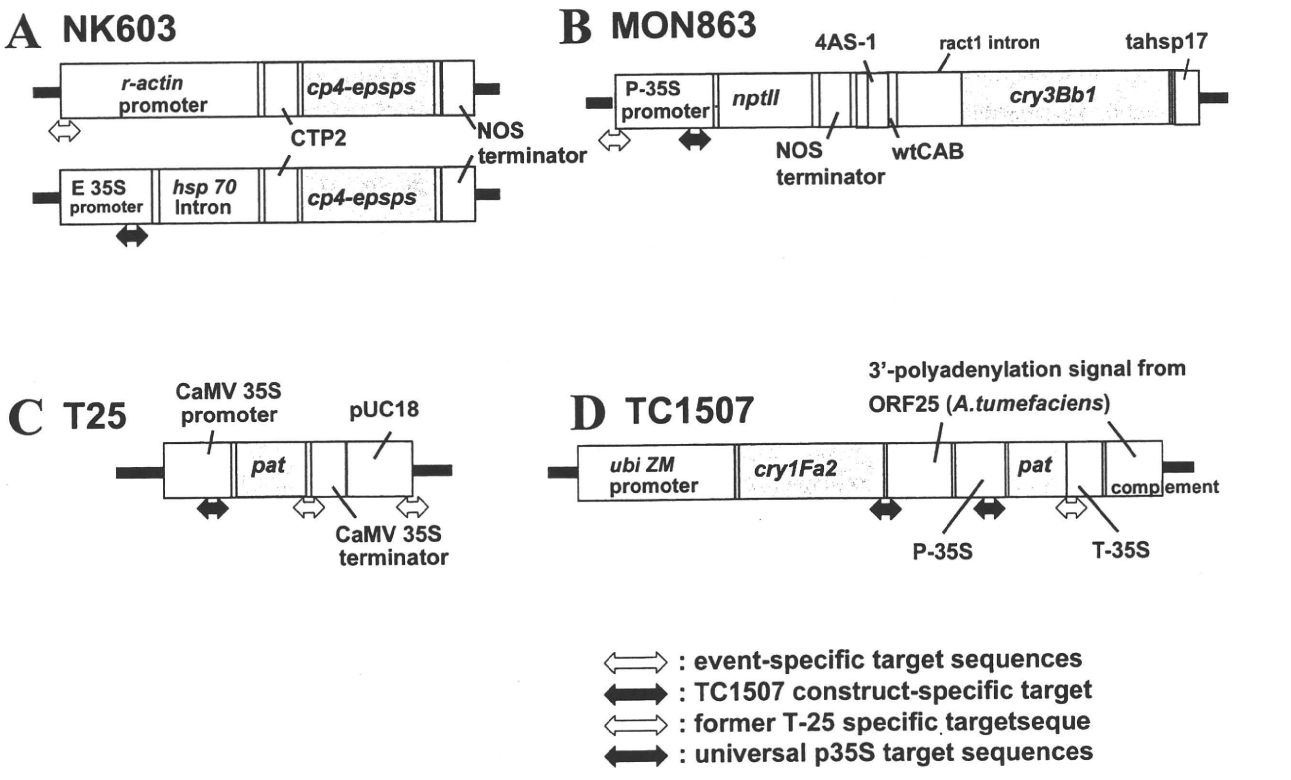

Fig. 1. Schematic diagrams showing the target positions in NK603 A, MON863 B, T25 C, and TC1507 D. Event-specific target sequences were designed for the flanking regions between the exogenous insert and the host maize DNA.

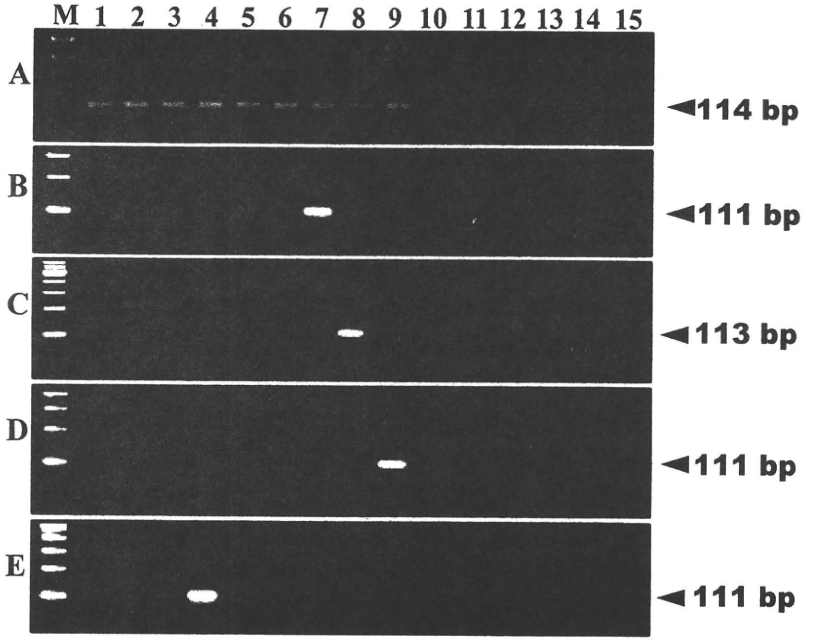

Fig. 2. Specificity tests for the designed primers. Agarose gel electrophoresis (3%) of the amplified PCR products corresponds to the 114-bp zSSIIb gene A, 111-bp MON863 B, 113-bp NK603 C, 111-bp TC1507 D and 111-bp T25 E. Arrowheads indicate the expected amplified products. Lanes 1-9, amplification of maize DNAs from non-GM maize, Bt11, GA21, T25, Event176, MON810, MON863, NK603 and TC1507, respectively; lanes 10-14, amplification of non-GM soy, Roundup Ready Soy, rice, wheat and barley, respectively; lane 15, no template control. M, molecular weight marker.

several control plasmids containing tandem insertions of GM-target and taxon-specific sequences for analytical calibrators have been developed (Kuribara *et al.*, 2002; Oguchi *et al.*, 2009). In this study, a novel calibrant plasmid, the pIIMul4v2.1, was developed for MON863, NK603, TC1507 and T25. The pIIMul4v2.1 has five segments: 151 bp of the *SSIIb* segment, 111 bp of the TC1507, 113 bp of the NK603, 111 bp of the T25 segment, and 111 bp of the MON863 segment. These segments were tandemly connected in this order and cloned into the vector (Fig. 3). We confirmed that unexpected amplifications were not observed under the present PCR conditions using the pIIMul4v2.1 as a template (data not shown).

Determination of Cf values for ABI PRISM 7700, 7900HT and 7500 We measured the Cf values for MON863, NK603, TC1507 and T25 with the ABI PRISM 7700, 7900HT and 7500 (Table 2). The average values from five laboratories for ABI PRISM 7700 and 7900HT, and three laboratories for 7500 were determined as experimental Cf values for the calculation of each GMO amount (%). For each event, the mean values with the three instruments were similar. The Cf values for MON863 and NK603 were closer to 0.5, which is the Cf value theoretically expected from F1 hybrid maize, including a single copy of the transgene per genome. Although comparing the RSD between the results from the ABI PRISM 7700 or 7900HT, and the results from 7500 is not simple because of the differences in sample numbers, the Cf value for T25 with the 7500 was also the largest of the four events, thus suggesting that the analytical results using the T25 method are relatively varied. As our previous method using the ABI PRISM 7700 (Kuribara *et al.*, 2002; Shindo *et al.*, 2002) has been adopted in the Annex of ISO 21570 (ISO, 2006), the 7700 was considered the standard model for our methods. However, we have employed the ABI PRISM 7900HT and 7500 as successors to the 7700 system and to expand the range of choices of instruments (Kodama *et al.*, 2002; Oguchi *et al.*, 2009). To evaluate the similarities between the 7700 and these two instruments, Student's *t*-test (α=0.05, 2-side) was performed (Fisher, 1954). *P* values of less than 0.05 in Table 2 indicate a statistically significant difference between the Cf values measured by the ABI PRISM 7700 and the other two instruments, which would suggest that the differences in the instruments would affect the measurement of the GM amounts.

Interlaboratory evaluation of the PCR quantification To evaluate the quantitative methods for the four GM maize events, MON863, NK603, TC1507 and T25, blind tests were performed with the participation of 16 laboratories for the

Fig. 3. Sequences (A) and schematic diagram (B) of the integrated fragments inserted into plasmid pIIMul4ver2.1. Primers and probes designed for the PCR system are indicated by open boxes and arrows, respectively.

Table 2. Experimental conversion factors used in this study.

	7700			7900HT			*P* value	7500			*P* value
	Mean	SD[a]	RSD[b]	Mean	SD	RSD	(comparison with 7700)	Mean	SD	RSD	(comparison with 7700)
MON863	0.54	0.010	1.85	0.52	0.010	1.92	0.0133	0.52	0.015	2.96	0.0376
NK603	0.50	0.013	2.71	0.54	0.012	2.27	0.634×10^{-3}	0.53	0.010	1.89	0.00940
TC1507	0.36	0.016	4.39	0.37	0.021	5.55	0.264	0.35	0.006	1.63	0.519
T25	0.35	0.045	12.7	0.39	0.023	5.84	0.115	0.37	0.020	5.41	0.591

[a] SD; Standard deviation
[b] RSD; Relative standard deviation

ABI PRISM 7700, and 15 laboratories for the 7900HT. Participants received primers, probes, and the extracted DNAs from 5 GM mixing levels for each event. The GMO amount (%) in each sample was calculated from the obtained data using Cf values in Table 2, and then Cochran outliers and Grubbs outliers were removed. Finally, statistical analyses were carried out as described previously (Kodama *et al.*, 2009; Shindo *et al.*, 2002), and the trueness and precision were obtained. The mean, bias (mean-value, %), repeatability RSD (RSD_r), and reproducibility RSD (RSD_R) at each mixing level and GM line were measured using both ABI PRISM 7700 and 7900HT (Table 3). In the results for the ABI PRISM 7700, the values of RSD_r and RSD_R at the level of 0.25% were both < 28%. At the level of 5.0%, the values of RSD_r and RSD_R were both < 13%. Generally, larger RSD_r and RSD_R values were observed for lower GM amounts. The ranges of obtained RSD_r and RSD_R here were similar to those previously reported for GMO events (Kodama *et al.*, 2009; Shindo *et al.*, 2002). However, the bias from three events, MON863, TC1507 and T25, particularly TC1507, appeared to be higher than their true values, even when compared with previously reported GM lines (Kodama *et al.*, 2009; Shindo *et al.*, 2002), suggesting the possibility that when using these quantitative methods, a slight overestimation of the amount of GM mixing levels occurs. The results obtained from the 5% samples were the most important to evaluate and should be acceptable because the threshold level of unintended comingling in Japan is 5%. In our previously established methods for GM maize, the bias values of quantitative results from 5% samples were < 25% (Kodama *et al.*, 2009; Shindo *et al.*, 2002). In Table 3, the bias values from 5% samples are acceptable, except for TC1507. The reason why the bias values

Table 3. Trueness and precision statistics for real-time quantitative PCR by ABI PRISM 7700 and 7900HT.

ABI PRISM 7700							ABI PRISM 7900HT						
		Trueness		Precision					Trueness		Precision		
		means	Bias			Detection limit			means	Bias			Detection limit
% (w/w)	Retained labs	GMO Amount, %	True value, %	RSD_r[a], %	RSD_R[b], %	Below 20 copies[c]	% (w/w)	Retained labs	GMO Amount, %	True value, %	RSD_r, %	RSD_R, %	Below 20 copies
MON863							**MON863**						
0.25	15	0.38	51.58	14.18	17.67	0/30	0.25	15	0.37	47.10	15.60	21.35	0/30
0.50	16	0.69	37.63	8.81	10.33	0/32	0.50	13	0.69	37.77	20.55	20.55	0/26
1.0	16	1.26	26.26	12.62	12.62	0/32	1.0	15	1.28	27.90	11.57	15.04	0/30
5.0	16	5.89	17.86	8.46	8.49	0/32	5.0	15	6.02	20.48	9.18	11.97	0/30
10.0	16	10.93	9.27	8.31	8.31	0/32	10.0	15	11.30	13.01	12.68	12.68	0/30
NK603							**NK603**						
0.25	15	0.25	-0.51	18.75	21.72	1/30	0.25	15	0.25	0.11	19.78	20.69	1/30
0.50	16	0.49	-1.95	19.43	25.04	0/32	0.50	14	0.54	7.80	15.13	19.06	0/28
1.0	14	1.02	2.19	5.55	7.53	0/28	1.0	15	1.03	2.75	17.67	17.67	0/30
5.0	16	4.84	-3.21	10.22	10.53	0/32	5.0	15	4.73	-5.31	8.93	10.74	0/30
10.0	16	9.06	-9.44	6.90	7.26	0/32	10.0	15	8.89	-11.10	15.53	15.53	0/30
TC1507							**TC1507**						
0.25	16	0.37	48.79	27.07	27.07	1/32	0.25	15	0.35	39.91	22.26	22.26	2/30
0.50	16	0.73	46.73	17.69	23.72	0/32	0.50	15	0.72	44.72	16.71	16.71	0/30
1.0	16	1.53	52.81	18.59	19.46	0/32	1.0	15	1.41	41.13	14.56	14.56	0/30
5.0	16	7.10	41.93	12.86	12.86	0/32	5.0	15	6.87	37.48	12.20	12.48	0/30
10.0	15	13.17	31.74	7.12	7.12	0/30	10.0	15	13.03	30.25	9.38	11.62	0/30
T25							**T25**						
0.25	15	0.34	36.44	25.84	25.84	3/30	0.25	15	0.29	16.15	25.69	27.84	5/30
0.50	15	0.65	30.84	17.57	17.86	0/30	0.50	15	0.58	15.78	20.57	24.99	0/30
1.0	16	1.32	32.23	13.29	15.12	0/32	1.0	15	1.11	11.24	10.77	18.08	0/30
5.0	16	6.02	20.39	10.66	12.12	0/32	5.0	15	5.20	3.94	11.76	20.70	0/30
10.0	14	11.39	13.94	9.60	10.76	0/28	10.0	15	9.95	-0.46	10.31	19.38	0/30

[a] RSD_r; 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.

from TC1507 were high is unclear at present. For application to practical monitoring of comingling of these GM events, further investigation is thus required.

In the results for the ABI PRISM 7900HT, all obtained data showed similar trends as the data obtained with the 7700. The values of RSD_r and RSD_R at the level of 0.25% were < 26% and < 28%, respectively, and the biases from TC1507 and T25 were also high, but the values were relatively low when compared with those from the ABI PRISM 7700. With regard to the limit of quantitation (LOQ), data below 20 copies cannot be reported with confidence because there was no calibrant below 20 copies. The measurement copies of the 0.25% samples were over 20 measurement copies, except in a few cases for T25, NK603, and TC1507. Therefore, it was estimated that the LOQ for MON863, NK603, TC1507, and T25 were all 0.25% for both the ABI PRISM 7700 and 7900HT.

Blind test using ABI PRISM 7500 The blind test was also conducted via a multilaboratory trial with the ABI PRISM 7500 for comparison with the results from the 7700 and 7900HT (Table 4). The trial was carried out with 3 laboratories in Japan. Materials such as primers and probes, and extracted DNAs were the same as for the ABI PRISM 7700 and 7900HT. The values of RSD_r and RSD_R at the level of 0.25% were both < 26%. A similar trend was observed with the ABI PRISM 7700 and 7900HT in terms of trueness, precision, and LOQ. These results suggest that the analytical results obtained with the ABI PRISM 7500 are comparable to those obtained from the 7700 and 7900HT.

Table 4. Trueness and precision by ABI PRISM 7500.

ABI PRISM 7500						
		Trueness		Precision		
		means	Bias			Detection limit
% (w/w)	Retained labs	GMO Amount, %	True value, %	RSD_r,[a] %	RSD_R,[b] %	Below 20 copies[c]
MON863						
0.25	3	0.35	39.98	13.46	13.46	0/6
0.50	3	0.66	31.57	19.62	19.62	0/6
1.0	3	1.22	21.51	7.69	8.43	0/6
5.0	3	5.99	19.87	3.25	3.25	0/6
10.0	3	11.64	16.44	10.30	10.68	0/6
NK603						
0.25	3	0.24	-2.38	7.01	13.45	0/6
0.50	3	0.45	-10.58	15.67	15.67	0/6
1.0	3	1.03	3.35	6.70	6.70	0/6
5.0	3	4.60	-7.96	2.19	3.30	0/6
10.0	3	8.76	-12.43	9.71	9.71	0/6
TC1507						
0.25	3	0.42	68.36	25.52	25.52	0/6
0.50	3	0.82	63.49	19.01	19.01	0/6
1.0	3	1.61	61.19	8.31	8.31	0/6
5.0	3	8.11	62.20	3.24	4.07	0/6
10.0	3	15.21	52.06	8.02	8.02	0/6
T25						
0.25	3	0.32	29.31	24.60	24.60	0/6
0.50	3	0.63	26.58	4.78	14.28	0/6
1.0	3	1.24	23.68	5.43	18.13	0/6
5.0	3	6.03	20.56	4.70	7.17	0/6
10.0	3	11.31	13.12	5.10	6.58	0/6

[a] SD; Standard deviation
[b] RSD; 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.

Conclusion

We developed specific quantitative methods for four GM-maize events, MON863, NK603, TC1507 and T25. First, the Cf values for each GM event were determined with three real-time PCR instruments, the ABI PRISM 7700, 7900HT and 7500. It was confirmed that the Cf values of both MON863 and NK603 are significantly different between the ABI PRISM 7700 and the other instruments, indicating that it is necessary to use individual Cf values for each instrument. Next, these analytical methods were evaluated in an interlaboratory study using two instruments, the ABI PRISM 7700 and 7900HT, and by a multilaboratory trial using the ABI PRISM 7500. The results obtained from the three instruments showed similar trends, and similar levels of trueness, precision, and LOQ. From these results, we concluded that these methods are applicable to all three instruments using individual Cf values for each instrument.

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調査・資料

リアルタイムPCRによるDNA検査に好適なポリプロピレンチューブの選択方法

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Selection of Suitable Polypropylene Tubes for DNA Testing Using Real-Time PCR

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Polypropylene microtubes (tubes) are generally used for bio-material tests in addition to PCR tests such as genetically modified organism (GMO) testings. However, the choice of suitable tubes is quite important, because it might influence the results: DNA binding and/or elution of chemical substances sometimes occurs. In this study, we established methods to select tubes with the most suitable characteristics for DNA testing.

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Key words: ポリプロピレンチューブ polypropylene tube; 遺伝子組換え体 genetically modified organism (GMO); DNA吸着 DNA binding; リアルタイムPCR real-time PCR

緒 言

ポリメラーゼ連鎖反応(PCR)は，臨床，環境および食品分野などのさまざまな検査に広く利用されており，食品分野では，遺伝子組換え食品(GMO)，食物アレルゲン，肉種鑑定，品種鑑定などの検査で用いられている．PCRをはじめとする生化学的検査は，従来から行われてきた化学分析とは異なり，比較的新しい分析技術であるため，検査法自体の精度管理手法が十分に確立されているとは言い難い．その反面，分析値の信頼性確保のために，ISOやCODEXなどの国際規格においては適切に分析精度を管理することが求められており，生化学的な試験についても一般に“試験所認定”と呼ばれるISO/IEC 17025認定の取得が進みつつある[1)~3)]．

我々は，リアルタイムPCRによるGMOの定量検査を行う際に，DNA溶液をある種のポリプロピレン(PP)製マイクロチューブ（チューブ）へ分注したところ，測定値が大きく変動する現象を見いだした．具体的には，PCRの鋳型となる検量線作成用標準プラスミドDNA溶液を特定のチューブに分注後，リアルタイムPCR法による測定を実施したところ，鋳型DNA溶液のコピー数が50 %以下に減少する事例が確認された．このコピー数の減少は分注後直ぐに生じたため，従来考えられてきたDNAの長期保存による分解の影響ではなく，分注後間もなく生じるチューブへのDNA吸着，あるいはチューブからのPCR阻害物質の溶出が原因であると推測された．また，武らもPP製品がPCRに影響を与える現象として，ガンマ線滅菌されたチューブから非水溶性の物質が溶出され，それが一定濃度以上であるとPCRを阻害することを報告している[4)]．

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このように，PCRなどのDNAに関する分析技術全般において，不適切なチューブを選択した場合には，検査時の測定結果に多大な影響を与える可能性があるため，チューブの選択にあたっては十分に留意する必要がある．

本検討において，我々はPCR検査の精度管理を目的として，DNA分析に好適なチューブを選択可能とするための手法を検討したので報告する．

実験方法

1. 試　料

1) PP製チューブ

本試験に用いたチューブは複数のメーカーから購入し，それぞれの同一ロット品を使用した．これらのチューブには同一メーカーから購入したものが一部含まれるが，DNAの低吸着性をうたった製品は含まれていない．チューブはTable 1に示したAからJ（計10種類）を本試験に供した．その内訳は，0.2 mL容量のPCR用チューブ3種，0.5から0.65 mL容量のチューブ2種，1.5から2.0 mL容量のチューブ5種である．チューブはすべて未滅菌の製品を入手し，121℃，0.12 Mpa，20分の条件でオートクレーブ滅菌後，70℃で乾燥したものを用いた．

2) DNA試料

紫外可視分光光度計によるO.D. 260 nmの測定試験には，同じ品質のDNAを安価に調製できる利点から，ニッポンジーン社よりColE1 DNAを購入した．購入したColE1 DNAは高濃度であったため，本検討にはO.D. 260 nmの測定値を基に，TE buffer（ニッポンジーン社製）で50 ng/μLに希釈してDNA濃度測定用ColE1 DNA試料を調製した．また，確認試験として実施したQuant-iT™ PicoGreen® dsDNA Assay Kit（ライフテクノロジーズ社製，以下PicoGreenKitと略す）によるDNA濃度の測定にもこれらを用いた．

2. チューブを評価するための測定方法

1) 紫外可視分光光度計によるO.D. 260 nm値の測定

O.D. 260 nmの吸光度はNano drop® ND-1000（ナノドロップテクノロジーズ社製）を用いて測定した．DNA濃度はO.D. 260 nmの値が1のDNA溶液を50 ng/μLとして算出した[5]．

2) Quant-iT™ PicoGreen® dsDNA Assay KitによるDNA濃度の測定

DNA濃度の測定操作はキットに添付のプロトコルに従い，蛍光測定装置はSpectra Max M2e-TS（日本モレキュラーデバイス社製）を用いた．検量線の作成にはキットに添付されているλDNAを希釈して調製した．

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*2 厚生労働省医薬食品局食品安全部通知“組換えDNA技術応用食品の検査方法について（一部改正）”平成20年度6月18日食安発第0618001号(2008)

Table 1. A list of purchased PP tubes

PP tubes	Volume (mL)
A	0.65[a]
B	0.50[a]
C	1.5[b]
D	1.5[a]
E	1.7[a]
F	2.0[b]
G	1.7[a]
H	0.20[c]
I	0.20[c]
J	0.20[c]

Ten kinds of PP tube were used in this study.
[a] PP Flatcap tubes
[b] PP Screwcap tubes
[c] PP PCR tubes

3) リアルタイムPCRによるCt値の測定

リアルタイムPCRによるCt値測定試験では，チューブへのDNA吸着と，チューブから溶出する物質がPCRに与える影響を明らかにするため，GMOにかかわる標準分析法で実績のあるGMダイズ(RRS)プラスミドセット—ColE1/TE—（ニッポンジーン社製）をCt値測定用プラスミドDNA試料として用いた．本検討では，このプラスミドセットのうち，ウェル間のCt値の変動が少なく比較的安定に測定可能な1,500, 20,000, 250,000コピー/2.5 μLの標準プラスミドをCt値測定用のプラスミドDNA試料として使用した[6],*1,*2．

Ct値の測定は，栗原らの方法[6]およびJAS分析試験ハンドブック*1の定量的PCR編に準じて，RRSに特異的な配列を標的とする定量PCRで行った．定量装置はABI PRISM® 7900HT（ライフテクノロジーズ社製）を用いた．リアルタイムPCRの反応液を調製するためのチューブは，後述の”紫外可視分光光度計によるO.D. 260 nmの測定試験”でDNA吸着およびO.D. 260 nmに吸光のある物質の溶出が確認されなかったチューブAを用いた．

3. チューブを評価するための測定用DNA試料の調製方法

手順の概略はFig. 1に示した．

試験は目的に応じて以下に示す二段階に分けて実施し，すべての測定は各3点併行で実施した．また，チューブに添加するDNA試料の液量は，供試するチューブ内側の面積差を考慮して，0.2 mLチューブでは10 μL,0.5 mLおよび0.65 mLチューブでは50 μL, 1.5 mLから2.0 mLのチューブでは100 μLとした．

第一段階は，“DNA濃度測定用ColE1 DNA試料”および“Ct値測定用プラスミドDNA試料”（以下，これらを合わせて測定用DNA試料とする）を各PP製チューブへ分注後，O.D. 260 nmの吸光度を測定して分注前と比較することでチューブへのDNA吸着，およびチューブからO.D. 260 nmに吸光のある物質が溶出する程度を調べた．

第二段階は，第一段階での評価から5種類のチューブ

を選択し，リアルタイム PCR により Ct 値を調べた．評価は，Threshold line (Th.) を 0.128 に固定した際の Ct 値を分注前の値と比較することで行った．

O.D. 260 nm の測定試験およびリアルタイム PCR による Ct 値測定試験に用いる DNA 試料は，Fig. 1 に示した条件 (1), (2) に従って調製した．供試するチューブはそれぞれの種類毎に各 11 本を用意した．

条件 (1) では 1 本のチューブに測定用 DNA 試料を添加・かくはん後，同一のピペットチップ（200 μL 用）を用いて同一のチューブ内で容量設定を変更することなく 10 回の吸引排出の操作を繰り返したものを測定用試料とした．

条件 (2) では，1 本目のチューブに 50 ng/μL に調製した測定用 DNA 試料を添加後，蓋を閉め，試料がチューブの壁面全体にいきわたるようボルテックスで数秒間かくはんした．チューブをスピンダウンして測定用 DNA 試料をチューブの底に集め，チューブへ測定用 DNA 試料を添加した際に使用したピペットチップで測定用 DNA 試料を回収し，2 本目のチューブに添加した．この作業を繰り返して 10 本目のチューブより回収し，条件 (2) の測定用試料とした．

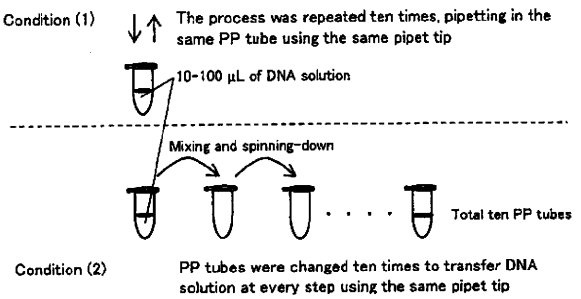

Fig. 1. Preparation of DNA samples for evaluating 10 kinds of tubes

Condition (1): DNA samples were prepared by pipetting ten times, using the designated volume of DNA solution and the same PP tube. The pipette tip was not changed.

Condition (2): DNA solution was added to a PP tube. After mixing with a touch mixer and spinning-down, DNA solution was transferred to the next fresh PP tube.

This preparation step was repeated a total of ten times in order to prepare DNA samples, without changing the pipette tip.

実験結果および考察

1. 吸光度による DNA 濃度測定結果の比較

A から J までの 10 種類のチューブに DNA 濃度測定用 ColE1 DNA 試料を添加し，条件 (1), (2) により調製した測定用試料について DNA 濃度を比較するとともに，チューブへの DNA 吸着およびチューブからの O.D. 260 nm に吸光のある溶出物質の有無を推察した (Table 2).

チューブ A, G および J については条件 (1) および (2) で DNA 濃度に顕著な差は認められなかった．他方，チューブ B, C, D, F, H および I は，いずれも条件 (2) において分注前の約 50 ng/μL から 35～40 ng/μL 程度へ DNA 濃度が低下した．このことから，各チューブへ DNA が吸着したと考えられた．また，チューブ E は条件 (2) において O.D. 260 nm 値が顕著に上昇していた．このためチューブ A, B と共に，夾雑物の影響を受けにくい 2 本鎖 DNA の濃度測定法である Pico Green Kit で再測定した．Pico Green Kit での測定値は O.D. 260 nm の測定値よりも低い値を示したが，これらは測定原理が異なる相対定量法で DNA 濃度を算出したことが主たる理由と考えられた (Table 3)．Pico Green Kit による測定の結果，チューブ A では分注の前後で DNA 濃度にほとんど変化がなかったが，チューブ B では吸光度による DNA 濃度測定結果と同様に DNA 量が減少していた．また，PicoGreenKit によるチューブ E での測定結果はチューブ A と同様に分注の前後で DNA 濃度にはほとんど変化が認められなかった．このことから，Table 2 のチューブ E における条件 (2) の処理で認められた，O.D. 260 nm の値から算出された DNA 濃度には，溶出物質由来の吸光が含まれていると示唆された．以上のことから，O.D. 260 nm の値から DNA 濃度を算出する際には，チューブからの溶出物質由来の吸光度が DNA の吸光度に加わって正確な測

Table 2. Comparison of DNA concentration from conditions (1) and (2)

	DNA concentration (ng/μL)		DNA concentration (ng/μL)
Tube A condition (1)	49.3	Tube F condition (1)	49.4
(2)	49.6	(2)	35.1
Tube B condition (1)	49.1	Tube G condition (1)	50.1
(2)	37.8	(2)	45.2
Tube C condition (1)	49.6	Tube H condition (1)	49.3
(2)	38.9	(2)	40.2
Tube D condition (1)	49.5	Tube I condition (1)	48.7
(2)	38.2	(2)	36.4
Tube E condition (1)	51.1	Tube J condition (1)	49.7
(2)	78.4	(2)	48.2

Numbers indicate ColE1 DNA concentration measured in terms of O.D. 260 nm, respectively.

Table 3. Comparison of DNA concentration using Quant-iT™ PicoGreen® dsDNA Assay Kit

	DNA concentration (ng/μL)	
	Condition (1)	Condition (2)
Tube A	30.7	30.0
Tube B	30.5	23.4
Tube E	30.7	30.7

After treatment under conditions (1) or (2) in Fig. 1, triplicate DNA solutions from PP tubes A, B and E were measured using Quant-iT™ PicoGreen® dsDNA Assay Kit, respectively. All numbers are averages of three results.

定結果が得られないことがあるため，留意すべきであると考えられた．

2. リアルタイム PCR による Ct 値測定試験結果の比較

チューブから溶出した物質が PCR を阻害・促進する可能性も併せて評価するため，Table 2 の結果から“DNA の吸着が少ないと思われたチューブ A”，“DNA が吸着したチューブ B, C および D”，“DNA 量が見かけ上増加したチューブ E”をそれぞれの代表例として選択し，リアルタイム PCR による Ct 値の測定試験を行った．リアルタイム PCR の反応液は，チューブのサイズごとに DNA 濃度測定用 ColE1 DNA 試料と同量の GM ダイズ (RRS) プラスミドセット—ColE1/TE—をチューブに添加後，条件 (1), (2) の処理を行い，Ct 値測定用プラスミド DNA 試料を調製した．Ct 値の測定は測定用試料 1 点あたり 3 反復で行い，リアルタイム PCR ソフトウェアにおいて Th.= 0.128 として解析し，評価を行った．その結果を Fig. 2 に示す．

条件 (1), (2) により調製した Ct 値測定用プラスミド DNA 試料について，リアルタイム PCR により得られた Ct 値をそれぞれ比較した結果，チューブ A, E では条件 (1), (2) において Ct 値に顕著な差は認められなかった．他方，O.D. 260 nm の測定試験で DNA の吸着が認められたチューブ B, C, D では，処理前のプラスミド DNA 試料と比べて DNA 増幅曲線の立ち上がりが遅れ，Ct 値が 1 から 3 程度増えた．Fig. 2 では，1,500 コピー/2.5 μL のプラスミド DNA 試料のみ Ct 値を数字で示したが，20,000 および 250,000 コピー/2.5 μL のプラスミド DNA 試料についても同様の傾向が認められた．リアルタイム PCR の増幅効率が 100% である仮定すると，理論上，Ct 値が 1 増加した場合には，鋳型 DNA 中に存在する標的 DNA の初期コピー数が 1/2 量であることに相当する．このことから，チューブ B, C, D における鋳型 DNA の初期コピー数は 1/2 から 1/8 であったと考えられる．また，Table 2 で示した O.D. 260 nm による測定試験で溶出物質の存在が認められたチューブ E については，チューブ A と同程度の Ct 値が得られ，PCR の阻害反応も観察されなかった．この結果から，チューブ E にはほとんど DNA が吸着しなかったことが再度確認され，O.D. 260 nm に吸光のある物質は溶出するが，これには PCR への顕著な阻害は認められないことが明らかとなった．

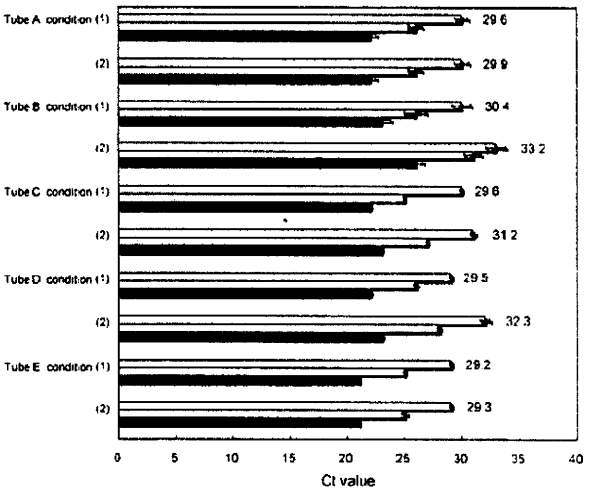

Fig. 2. Comparison of Ct values under conditions (1) and (2) in Fig. 1

Ct values from 1,500, 20,000 and 250,000 copies/2.5 μL of GM Soy Detection Plasmid in the case of Th.=0.128 are shown.
White bars, light gray bars and dark gray bars are Ct values of 1,500, 20,000 and 250,000 copies/2.5 μL of plasmid DNA, respectively.
Ct values for 1,500 copies of plasmid DNA are averages of three results.
Error bars: standard deviation of values from triplicate DNA solutions.

以上，“1. 吸光度による DNA 濃度測定結果の比較”および“2. リアルタイム PCR による Ct 値測定試験結果の比較”で評価した場合には，少なくとも両試験で良好な結果を示したチューブ A が DNA 検査には最も好適と判断された．

ま と め

PP 製チューブに起因する DNA の吸着事例を見いだし，DNA 分析に好適なチューブを選択可能とするための手法を開発した．チューブへの DNA 吸着およびチューブからの O.D. 260 nm に吸光のある物質の溶出程度は，ColE1 DNA 溶液を用いた O.D. 260 nm の吸光度測定による簡易な検定法で評価が可能と考えられた．さらに，チューブへの DNA 吸着とチューブから溶出した溶出物の影響が相殺されてしまう場合においても，まず吸光度測定試験で吸着および溶出が確認されなかったチューブを選び，これらをリアルタイム PCR による Ct 値の測定試験に供することで，PCR 検査に好適なチューブを選別できた．

本報告で示したチューブの評価法は，PCR に好適なチューブを選択するための有効な 1 つの手段となりうる．本検討結果は，先に述べた武らの報告[4]の検討結果と併せて，PCR 検査だけでなく PP 製のチューブの質が問われる DNA 検査全般にわたる精度管理に役立つものと期待される．

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Development and Evaluation of Event-Specific Quantitative PCR Method for Genetically Modified Soybean A2704-12

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A novel real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) soybean event; A2704-12. During the plant transformation, DNA fragments derived from pUC19 plasmid were integrated in A2704-12, and the region was found to be A2704-12 specific. The pUC19-derived DNA sequences were used as primers for the specific detection of A2704-12. We first tried to construct a standard plasmid for A2704-12 quantification using pUC19. However, non-specific signals appeared with both qualitative and quantitative PCR analyses using the specific primers with pUC19 as a template, and we then constructed a plasmid using pBR322. The conversion factor (C_f), which is required to calculate the amount of the genetically modified organism (GMO), was experimentally determined with two real-time PCR instruments, the Applied Biosystems 7900HT and the Applied Biosystems 7500. The determined C_f values were both 0.98. The quantitative method was evaluated by means of blind tests in multi-laboratory trials using the two real-time PCR instruments. The limit of quantitation for the method was estimated to be 0.1%. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSD_R), and the determined bias and RSD_R values for the method were each less than 20%. These results suggest that the developed method would be suitable for practical analyses for the detection and quantification of A2704-12.

Key words: A2704-12; event-specific; genetically modified (GM); real-time PCR; soybean

Introduction

The use of genetically modified (GM) crops has been increasing since their commercialization in 1996. After more than a decade, the global area of GM crops has increased approximately 80-fold, from 1.7 million hectares in six countries in 1996, to 134 million hectares in 25 countries in 2009[1)]. The utilization of GM crops has generated substantial economical benefits, but, nevertheless, has been subjected to rigid control. The presence of GM products in crops or foods is obliged to be labeled in the European Union (EU), Korea, Japan, Australia, and many other countries. In Japan, the genetically modified organism (GMO) labeling system has been defined by the "JAS law"*1 and the "Food Sanitation law"*2, and the thresholds for the unintentional commingling level for approved GM soy and maize were both set at 5%[2),*3,*4].

The Japanese food self-sufficiency ratio on a calorie supply basis has been hovering around 40% in recent years, but the ratios among grains, except for rice, are extremely low*5. Among them, soybeans are one of the most important crops in Japan. The domestic consump-

*1 Notification No. 1173 (Oct. 1, 2007); Ministry of Agriculture, Forestry and Fisheries of Japan (2007)

*2 Notification No. 79 (Mar. 15, 2001); Ministry of Heath, Labour and Welfare of Japan (2001)

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

*4 Notification No. 517 (Mar. 31, 2000); Ministry of Agriculture, Forestry and Fisheries of Japan (2000)

*5 Annual Report on Food, Agriculture and Rural Areas in Japan FY2006 Policies on Food, Agriculture and Rural Areas in Japan FY2007, Summary of Ministry of Agriculture, Forestry and Fisheries of Japan; http://www.maff.go.jp/e/pdf/fy2006_rep.pdf

tion of soybeans as oil and food is over 4 million tons/year, but the self-sufficiency ratio for this crop is only approximately 5%*6, *i.e.*, more than 90% of soybeans are imported into Japan. Most of them are imported from the United States, where GM soybeans account for more than 90% of the soybean cultivation areas[1]. There are several approved GM soybean events in Japan*7. The first commercial GM soybean was a glyphosate-tolerant soybean (GTS), event 40-3-2 [Roundup Ready® soybean (RRS)]. RRS has received regulatory approval in many countries, including Japan. Next, glufosinate-tolerant soybeans such as A2704-12 and A5547-127 were approved, and then the second generation of GTS, MON89788, was recently approved.

A2704-12 and A5547-127 contain the same transgene cassette consisting of cauliflower mosaic virus 35 S promoter (P35S), the synthetic *pat* gene which codes phosphinothricin *N*-acetyltransferase derived from *Streptomyces viridochromogenes*[3,4], and cauliflower mosaic virus 35S terminator (T35S)[5,6]. Many methods for analyzing RRS and MON89788 have been published[7-11], but there is no report on quantification of A2704-12, although A2704-12 is now being commercially cultivated.

In this study, we developed a new quantitative method for A2704-12, using event-specific PCR, and then evaluated the method with two multi-laboratory trials. The development of the quantitative method was mainly carried out in the National Food Research Institute, and the interlaboratory collaborative study was independently conducted with the National Institute of Health Sciences (NIHS).

Materials and Methods

Plant materials

Regarding soybean and maize seeds, A2704-12 and a non-GM isoline of A2704-12 used as a non-GM soy sample were kindly provided by the developer, MON 89788, RRS, MON810, MON863, MON88017, and NK 603 by Monsanto Company (St. Louis, MO, USA), Bt11, Event176, GA21, and MIR604 by Syngenta Seeds AG (Basel, Switzerland), TC1507 and DAS59122 by Pioneer Hi-Bred International (Johnston, IA, USA), 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 rice (*Oryza sativa*) variety Kinuhikari, wheat (*Triticum aestivum*) variety Haruyutaka, and barley (*Hordeum vulgare*) variety Harrington were harvested in Japan. A5547-127 was purchased from the American Oil Chemists' Society (AOCS) (Urbana, IL, USA) as DNA extract (AOCS 0707-C; above 999.9 ng/μL of GM DNA).

Oligonucleotide primers and probes

For the specific detection of A2704-12, a pair of primers (KVM175; 5′-GCAAAAAAGCGGTTAGCTCCT-3′ and SMO001; 5′-ATTCAGGCTGCGCAACTGTT-3′) and a fluorescent dye-labeled probe (TM031; 5′-CGG-TCCTCCGATCGCCCTTCC-3′) were used for real-time PCR. The sequences of these primers and probe were taken from the report of the European Commission's Joint Research Centre (JRC, IRMM, Retieseweg, Geel, Belgium)*8. *Le1*[12] was used as a soybean-specific endogenous reference DNA for quantitative analysis. For specific detection of *Le1*, the primers (Le1n02-5′; 5′-GCCCTCTACTCCACCCCCA-3′ and Le1n02-3′; 5′-GCCCATCTGCAAGCCTTTTT-3′) and fluorescent dye-labeled probe (Le1-Taq; 5′-AGCTTCGCCGCTTCCTT-CAACTTCAC-3′) were used[8]. 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

Specific sequence fragments from A2704-12 and the endogenous soybean *Le1* gene were synthesized as a single oligonucleotide in tandem and inserted into a pBR322 vector. The constructed plasmid was purified by equilibrium centrifugation in a CsCl gradient to collect the covalently closed circular DNA, which was linearized by cutting at a restriction site located outside the integrated fragment. The concentration of the linearized DNA was calculated from the ultraviolet (UV) absorbance measured with a spectrophotometer, DU 800 (Beckman Coulter, Fullerton, CA, USA) as described previously[8], and converted to the molar concentration. The 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, Tokyo, Japan) solution. Finally, the copy numbers of the diluted plasmids were adjusted based on the *Le1* segment of pMulSL2, which has been adopted in the Japanese standard analytical method*9, using quantitative real-time PCR analyses.

*6 Ministry of Agriculture, Forestry and Fisheries of Japan; http://www.maff.go.jp/j/seisan/ryutu/daizu/d_data/pdf/011_juyou.pdf

*7 List of products that have undergone safety assessment and been announced in the Official Gazette (May 12, 2010) of the Department of Food Safety, Ministry of Health, Labour, and Welfare; http://www.mhlw.go.jp/english/topics/food/pdf/sec01-2.pdf

*8 Event-specific method for the quantification of soybean line A2704-12 using real-time PCR; http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_validated_Method.pdf

*9 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

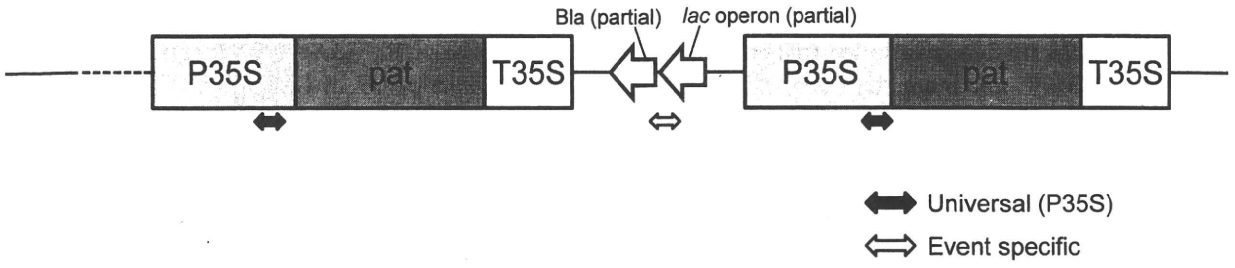

Fig. 1. Schematic diagrams of the target positions in A2704-12
The event-specific target sequence of A2704-12 is the junction region of two partial sequences derived from the *bla* gene and *lac* operon, corresponding to positions 248–267 and 2036–2056 in pUC19, between the two *pat* gene cassettes. The nucleotide sequence of pUC19 is available under accession number L09137.

Preparation of test samples and DNA extraction

To evaluate the quantitative method, we used six mixing levels of test materials containing 0%, 0.10%, 0.50%, 1.0%, 5.0%, and 10.0% A2704-12. To prepare the mixed samples, we ground A2704-12 seeds and non-GM seeds using MM200 and ZM100 grinders (Retsch, Haan, Germany), respectively, as described previously[13], and then mixed the samples on a weight-to-weight basis. DNA was extracted from the ground materials using GM quicker (NIPPON GENE) according to the manufacturer's manual. The concentration and quality of extracted DNA solutions were evaluated by measuring UV absorbance with a spectrophotometer, ND-100 (NanoDrop Technologies, Wilmington, DE, USA). Soybean genomic DNA solutions were adjusted to a concentration of 20 ng/μL.

Qualitative PCR

Qualitative PCR using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies), and agarose gel electrophoresis were performed as described previously by Kuribara *et al.* (2002).

Quantitative PCR

TaqMan® real-time PCR assays were carried out using the Applied Biosystems 7900HT (AB 7900) or the Applied Biosystems 7500 (AB 7500) (Life Technologies), in 25 μL final volume, containing 50 ng of sample DNA, 12.5 μL Universal Master Mix (Life Technologies), 0.5 μM primer pairs, and 0.2 μM probe. The step-cycle program was as follows: 2 min at 50℃, 10 min at 95℃, 45 cycles, 30 s at 95℃, and 1 min at 59℃. In the reaction plate, each sample was measured in triplicate.

Multi-laboratory trial

Multi-laboratory trials were performed with the AB 7900 and the AB 7500 independently and consisted of 2 separate stages: measurement of the C_f value and a blind test. All measurements were conducted by 5 laboratories for the AB 7900 and 3 laboratories for the AB 7500. Experimental protocols were provided by the NIHS. Quantitative real-time PCR was performed with primers, probes, Universal Master Mix, and blind DNA solutions supplied by NIHS.

The first stage was the experimental determination of the C_f 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 C_f value for A2704-12, 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 C_f 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 soybean genomic DNAs extracted from 6 different concentrations of A2704-12, 0%, 0.10%, 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 means of Cochran's test[14] and Grubbs' test[15), 16].

Results and Discussion

Specificity of the PCR system for A2704-12

A2704-12 contains two copies of the *pat* gene cassette inserted in a head-to-tail configuration*[10]. The biotic transformation of soybeans was conducted by microparticle bombardment with a pUC19-based plasmid containing the *pat* gene cassette. The pUC19 plasmid contains an antibiotic resistance gene; *beta-lactamase* (*bla*), and a *lac* operon, and several fragments derived from pUC19 DNA exist in the A2704-12 genome. Partial sequences derived from the *bla* gene and *lac* operon are integrated side-by-side between the two *pat* gene cassettes*[10], and this site is unique to A2704-12. For specific detection of A2704-12, the junction site was used (Fig. 1). The specificity of the primer set was confirmed by qualitative PCR. The expected 64-bp product was detected using genomic DNA solutions from A2704-12, but not from non-GM soybeans; GM soybeans RRS, MON89788, and another glufosinate-tolerant soybean: A5547-127; non-GM maize; 11 lines of GM maize; rice, wheat, and barley; and the no template control (Fig. 2).

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

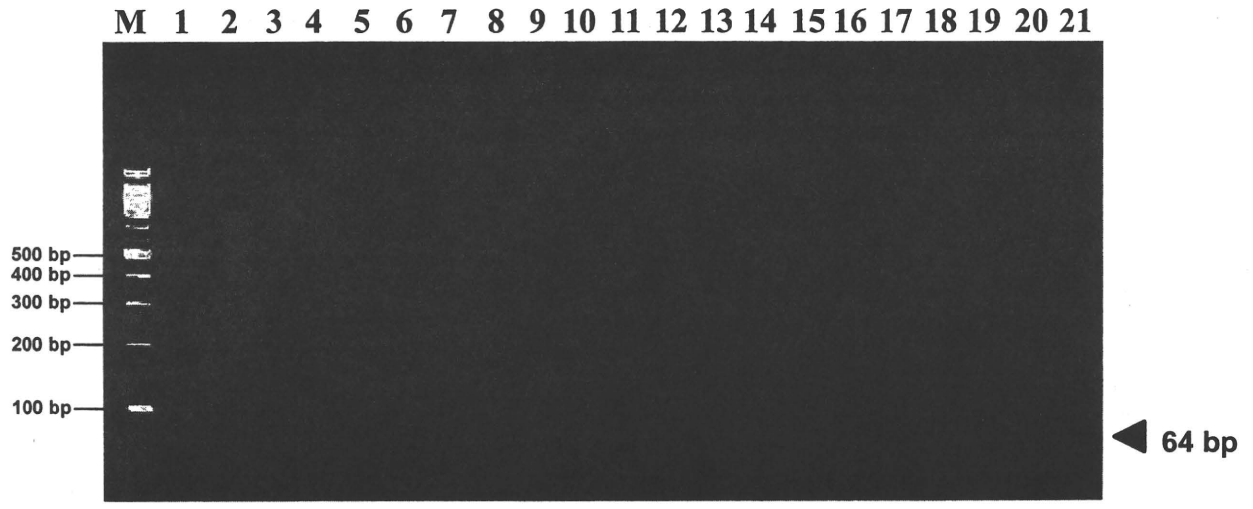

Fig. 2. Specificity test of the designed primer pair for genomic DNAs from several GM events or crops
Agarose gel (3.0%) electrophoretogram of the amplified PCR products corresponding to the 64 bp of A2704-12 DNA. Arrowhead indicates the expected amplified product. Lanes 1–11, eleven GM maize events, namely NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MIR604, MON88017, DAS59122, and MON863, respectively; 12 and 13, non-GM maize and non-GM soy, respectively; 14–17, four GM soybean events, namely, RRS, MON89788, A2704-12, and A5547-127, respectively; 18–21, rice, wheat, barley, and no template, respectively. Lane M shows 100 bp ladder size markers.

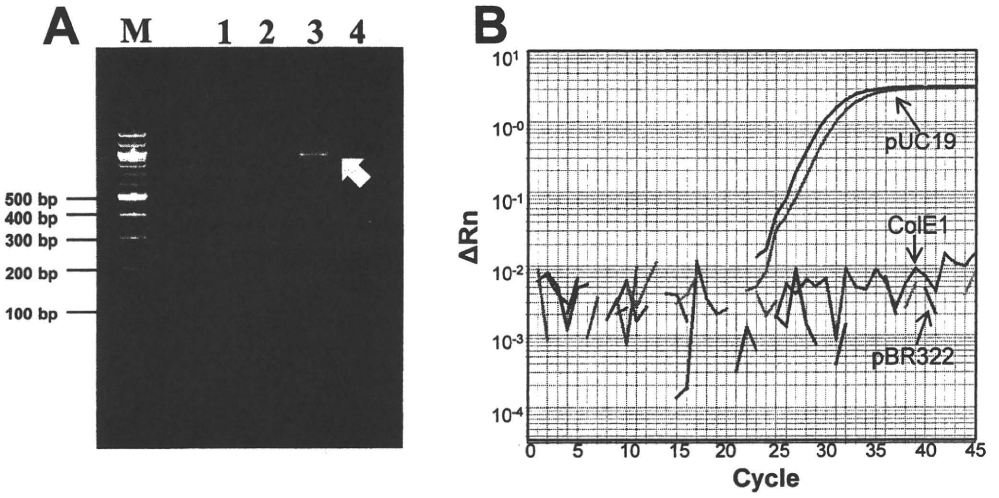

Fig. 3. Specificity test of the designed primer pair and probe for plasmid DNAs
A: Qualitative PCR analysis. Lanes 1–4, A2704-12 genomic DNA, ColE1 DNA, pUC19, and pBR322, respectively. The arrow indicates a non-specific amplification product. Lane M shows 100 bp ladder size markers.
B: Quantitative PCR analysis. pUC19-, pBR322-, or ColE1-derived signals in the amplification profile using the AB 7900 are shown. Concentrations of DNA solutions of A2704-12 genomic DNA, ColE1 DNA, pUC19, and pBR322 were 20, 5, 10, and 10 ng/μL, respectively.

Construction of calibrant plasmid for A2704-12

In Japan, one of the features of standard quantitative methods is the utilization of standard plasmid DNA as reference molecules and calibrators. We have developed several standard plasmids[8), 17)] using pUC19 or pBR322. Although pUC19 is a useful plasmid containing a high-copy-number replicon[18)], when the pUC19 plasmid was used as a template, a non-specific amplified product of around 1.0 kb was observed (Fig. 3A). Furthermore, a non-specific signal was detected by quantitative PCR analysis (Fig. 3B). As mentioned above, for the specific detection of A2704-12, the pUC19-derived sequences, KVM175 and SMO001, which are a part of the sequences of *bla* gene and *lac* operon, respectively, were used as primers. The unexpected band may have been caused by the sequences of these primers, indicating the possibility that inaccurate quantification could occur if pUC19-based plasmids were used as a calibrator for quantification. Thus, we used pBR322 plasmid for the construction of the standard plasmid, designated as pLLS. To prepare the pLLS plasmid, we removed a *bla* gene partial sequence that included KVM175 from pBR 322 and then inserted the A2704-12 and *Le1* segments (Fig. 4). Figure 5 shows the amplification plots of the pLLS plasmids diluted to a concentration ranging from 20 to 250,000 copies per reaction (Fig. 5A and B). The *R*

^{2} values from both A2704-12 and *Le1* were above 0.999 (Figs. 5C and D), which is an acceptable level (R^2 should be above 0.990)[8]. The slopes, representing the amplification efficiencies, were −3.44 and −3.32 for A2704-12 and *Le1* standard curves, respectively. PCR efficiency was calculated by use of the following equation: PCR efficiency $=10^{(-1/\text{slope})}$[19]. The theoretical value with an efficiency of 100% in each cycle is 2.00, and, in this study, PCR efficiencies of 1.95 and 1.99 were obtained for A2704-12 and *Le1*, respectively.

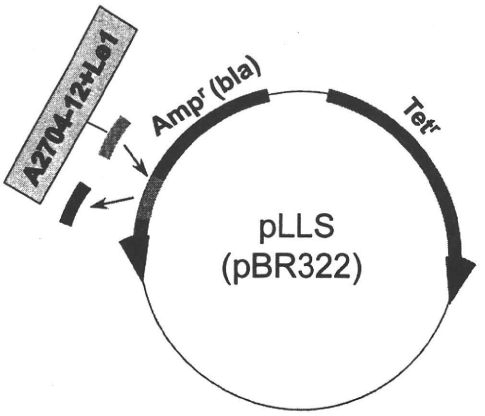

Fig. 4. Schematic diagram of the construction of pLLS plasmid
The region corresponding to positions 3613–3843 in pBR322 was removed, and the specific sequences of A2704-12 and the *Le1* were inserted in tandem. The nucleotide sequence of pBR322 is available under accession number J01749.

Determination of the C_f value for A2704-12

To determine the experimental C_f value for A2704-12, we measured the copy numbers of *Le1* and A2704-12 in the extracted DNA from A2704-12 seeds. The C_f value was determined with two real-time PCR instruments (the AB 7900 and the AB 7500) independently, from the results of 5 laboratories for the AB 7900 and 3 laboratories for the AB 7500. The measurement was repeated twice, and the C_f value was determined as the mean of values measured by these laboratories. The determined C_f values with the AB 7900 and the AB 7500 were similar; in fact, they took the same value of

Table 1. Experimental conversion factor for A2704-12

7900			7500		
Mean	SD	RSD	Mean	SD	RSD
0.98	0.0423	4.31	0.98	0.0232	2.36

SD: Standard deviation
RSD: Relative standard deviation

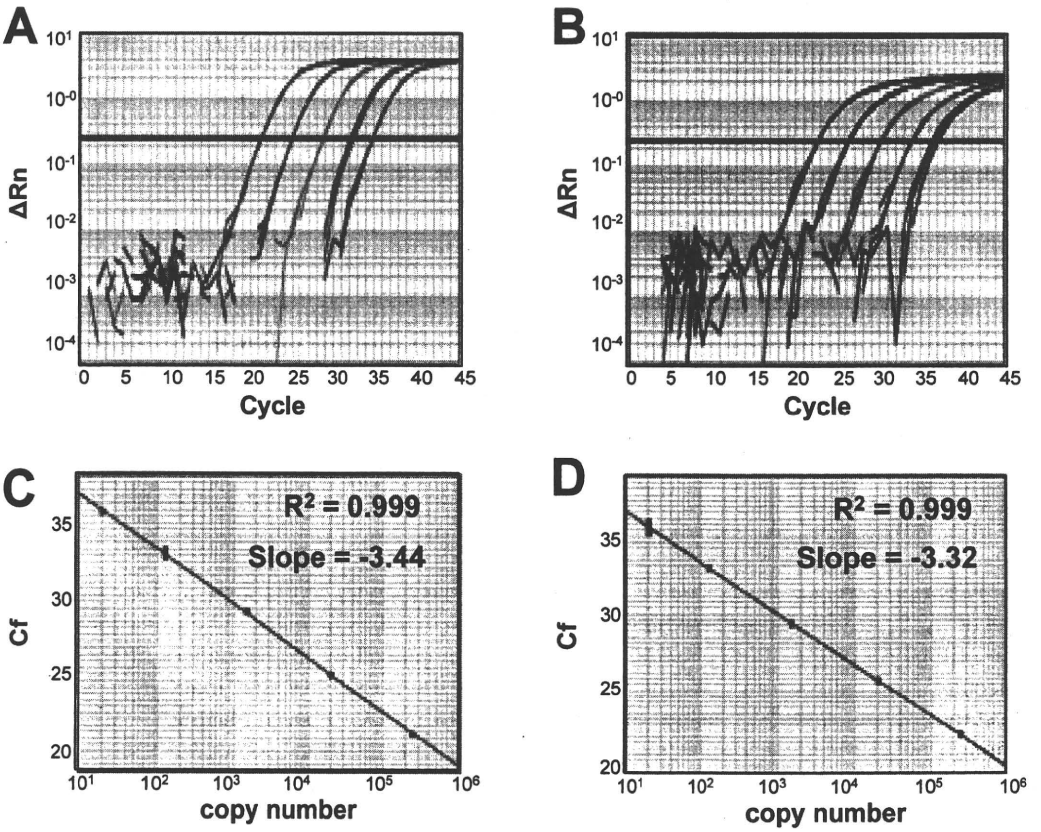

Fig. 5. Amplification plots for dilution series of pLLS plasmid and corresponding curves
Typical amplifications of A2704-12 and *Le1* segment are shown in A and B, respectively. The respective standard curves are shown in the panel below, and the R^2 and slope values of each standard curve are indicated at the upper-right corners. The dilutions contained 250,000, 20,000, 1,500, 125, and 20 initial template copies per reaction, respectively. These analyses were performed with the AB 7900.

Table 2. Summary of accuracy and precision statistics for real-time PCR by ABI PRISM 7900HT and 7500

7900HT	Retained labs	Trueness		Precision		Detection limit
		Means	Bias	RSD_r[a], %	RSD_R[b], %	Below 20 cipies[c]
% (w/w)		GMO Amount, %	True value, %			
0.10	5	0.101	1.0	9.4	9.9	0/10
0.50	5	0.455	−9.0	6.4	7.5	0/10
1.0	5	0.919	−8.1	7.7[d]	7.7[d]	0/10
5.0	5	4.27	−14.6	9.9[d]	9.9[d]	0/10
10.0	5	8.96	−10.5	10.7	10.7	0/10
7500	Retained labs	Trueness		Precision		Detection limit
		Means	Bias	RSD_r[a], %	RSD_R[b], %	Below 20 cipies[c]
% (w/w)		GMO Amount, %	True value, %			
0.10	3	0.112	11.7	11.0	12.1	0/6
0.50	3	0.503	0.7	13.2[d]	13.2[d]	0/6
1.0	3	0.992	−0.8	3.0	6.7	0/6
5.0	3	4.71	−5.8	8.2[d]	8.2[d]	0/6
10.0	3	9.30	−7.1	3.3	4.0	0/6

[a] RSD_r: Repeatability relative standard deviation
[b] RSD_R: Reproducibility relative standard deviation
[c] Number of values less than 20 copies per the total number of retained data.
[d] When RSD_r was above RSD_R, RSD_R was considered to be the same as RSD_r[24].

0.98 rounded to the nearest hundredth of a unit (Table 1). From these results, we used 0.98 in the following quantifications as the common C_f value for the two instruments.

Evaluation of the PCR quantification by multi-laboratory trials

We performed multi-laboratory trials to evaluate the developed quantitative method for A2704-12 as a blind test using the AB 7900 and the AB 7500 instruments in 5 and 3 laboratories, respectively. We used DNA solution-based blind samples as described previously[20), 21)]. For PCR quantification, analytical procedures would be divided into two main steps, that is, DNA extraction from samples and real-time PCR measurements. In our previous study, the Japanese standard analytical methods*9 which were developed to quantify RRS and several GM maize events, were validated with an interlaboratory study which consisted of 2 steps, namely, DNA extraction and PCR quantification[22)]. After that, the established methods were evaluated with another interlaboratory study which was performed using the same materials without the DNA extraction step[23)]. However, the obtained precisions from the two studies were almost the same, suggesting that, at least in our system, the repeatability of relative standard deviation (RSD_r), and reproducibility of relative standard deviation (RSD_R) of the DNA extraction step were significantly smaller than those of the PCR quantification step. It was also suggested that the relatively large RSD_r and RSD_R values which were obtained from GMO quantification, may be attributed to the principle of real-time PCR, which is based on relative quantification between target and taxon-specific sequences, rather than absolute quantification[23)].

All the participants received primers, probes, and test samples consisting of six different concentrations of A 2704-12, and the measurement was performed twice. All the submitted data were examined for outlier laboratories with extreme variation using Cochran's test ($p<0.025$) and with an extreme average level using Grubbs' test ($p<0.025$) for the AB 7900 and the 7500 independently, as previously described[22), 23)], and no outlier was found. The blank sample, 0% A2704-12, was used to estimate invalid laboratories, and no laboratory was eliminated. We then used all of the submitted data obtained from mixed samples with five different GM contents (0.1%, 0.5%, 1.0%, 5.0%, and 10.0% concentrations) for further statistical analyses. The trueness and precision were determined for the AB 7900 and the 7500 as previously described[22), 23)]. The mean, bias (mean-value, %), RSD_r, and RSD_R of blind samples were measured (Table 2). The determined bias, RSD_r, and RSD_R for the AB 7900 ranged from −14.6% to 1.0%, from 6.4% to 9.9%, and from 7.5% to 10.7%, respectively. The determined bias, RSD_r, and RSD_R for the AB 7500 ranged from −7.1% to 11.7%, from 3.0% to 13.2%, and from 4.0% to 13.2%, respectively. The obtained bias, RSD_r, and RSD_R here were similar to or within a narrower range than those in previously reported GMO events[22), 23)]. The data below 20 copies were extrapolated from the standard curve in our method because there was no calibrant below 20 copies. In Table 2, all the measured copy numbers of 0.10% samples were over 20 copies. Therefore, we estimate that the limit of quantitation (LOQ) for A2704-12 is 0.10% in this method.

We developed a specific quantification method for

GM soybean A2704-12. The experimentally determined C_f value was 0.98. The LOQ, trueness, and precision of this method were similar to or better than those of previous methods[20)-23)]. ISO 24276 specifies the LOQ in GMO analysis, and the values are generally observed to have a RSD_R of 25% or less at the lowest level*[11], all the RSD_R obtained in this study met this criterion. Therefore, we concluded that the developed method would be applicable for the detection and quantification of A2704-12 to monitor the validity of the food labeling system in Japan.

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