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Study of PCR Detection Methods for Genetically Modified Soybeans with Reference Molecules

HSU-YANG LIN[1], HSIU-AN WEI[1], FU-PANG LIN[2] AND DANIEL YANG-CHIH SHIH[1]*

[1] Bureau of Food and Drug Analysis, Department of Health, Executive Yuan, 161-2, Kunyang St., Taipei 115, Taiwan, R.O.C.

[2] Institute of Bisocience and Biotechnology, National Taiwan Ocean University, 2, Pei-Ning Rd., Keelung 202, Taiwan, R.O.C.

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ABSTRACT

The study of detection methods for genetically modified (GM) soy using certified reference material (CRM) and novel references molecules was operated on LightCycler real-time PCR machines system. The test results of this study demonstrated the methods used to be applicable to the specific quantitation of one line of GM soy. Independent repeat tests for 5, 2 and 1% CRM were 17, 11 and 11, and the test results mean ± SD were 4.89 ± 0.45, 1.98 ± 0.63 and 1.09 ± 0.05, respectively. Series 6 repeat test by use of CRM and references molecules, the results of CRM test were 5.45 ± 0.32, 2.42 ± 0.13 and 1.20 ± 0.09 (mean ± SD), and 4.83 ± 0.45, 2.09 ± 0.12 and 1.08 ± 0.10 for references molecules testing, to 5, 2, and 1% GM content, respectively. Further, soymilk was not detected by the ELISA method at OD_{450} when boiled under 70°C, 1~3 min but high to 100°C was detected by PCR method. Over 10 min under 121°C, DNA highly degradation detection was more difficult. Testing results should help support the practical detection for the GM soy.

Key word: real-time PCR, PCR, ELISA, reference molecules, GMO, soybeans, quantification

INTRODUCTION

Gene cloning and manipulating techniques are being increasingly exploited. Many genetically modified crops which are insect resistant and herbicide tolerant have been successfully developed and applied to field planting. USA is the most advanced country in this field. More than 50 genetically modified crops have been authorized for commercial production. According to the information from OECD[(1)], the most popular genetically modified crops tested in the field are soybean, tomato, maize, potato, wheat, cotton, sugar beet, rapeseed, and tobacco. In Taiwan, genetically modified papaya, tomato and rice have been tested in the field and evaluated. The main purpose of genetically modified crops is to alter the characteristics of crops. According to purposes, these crops can be classified to several categories: Firstly, the quality of products is altered, such as color, maturation-delayed, high starch-contained, and carotenoid-contained. Secondly, crops are insect resistance, such as moth resistance cotton, virus resistance tobacco, and insect resistance maize. Crops have special agricultural characteristics in planting, such as drought-tolerant maize, and herbicide tolerance soybean and maize. Thirdly, crops have other characteristics, such as heavy-metal-tolerance. New generations of genetically modified crops which have medical effects such as vaccine for anti-enterovirus are currently under study and development. The statistical information related to the planting of genetically modified crops are also growing tremendously. Planting areas of genetically modified crops are mainly in America, and about 70% of them are in USA (The information from Asia is not included)[(2)].

Up to 2005, 5 species of genetically modified soybeans around the world have been past the safety evaluation and then allowed to be sold on the market[(1)]. The major characteristics of genetically modified soybean are herbicide-tolerance and high oleic acid content. More characteristics under development include low saturated fatty acid content and high stearic acid content. According to the statistical information from the Council of Agriculture of the Executive Yuan, there are around 200 tons of soybeans are imported to Taiwan every year. About 95% of them are imported from USA. In Taiwan, it is estimated that about 50% of the soybean on the market are genetically modified soybean[(2)]. Detection methods for genetically modified food are classified to: Firstly, nucleotide-based amplification methods, including polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), fingerprinting techniques (RFLP, AFLP, RAPD, etc.), probe hybridization, self-sustained sequence replication (SSR), and Q replicase amplification. Secondly, protein-based methods, including one-dimensional SDS gel electrophoresis, two-dimensional SDS gel electrophoresis, Western-blot analysis, and ELISA. Thirdly, detection of enzymatic activities[(2-6)]. Each method has its specificity, merits, and defects. Generally, detection methods for fresh materials have fewer problems. As for processed products, enzymatic methods are inadequate because the protein has probably denatured. As for some highly processed food, PCR methods are also

* Author for correspondence. Tel: +886-2-26531265; Fax: +886-2-26531268; E-mail:shihdyc@nlfd.gov.tw

inadequate because the DNA fragments have probably broken into much smaller pieces. Among three categories of methods described above, PCR methods are the most widely used. Each laboratory can design different primers for detection based on the inserted regulatory sequence or structural gene. Based on characteristics of primers selected, PCR methods are classified to screen methods and product-specific detection methods. Products obtained from PCR methods can be tested by confirmation methods such as nucleotide sequencing, nucleotide endodigested fingerprinting, and probe hybridization. PCR methods can be used for qualitative analysis as well as quantitative analysis[7-10]. For the regulation of the genetically modified food labeling system, countries in Europe are aggressively developing qualitative and quantitative PCR to solve problems related to the detection of genetically modified food. PCR-based methods have been cited and approved by many sources. Real-time PCR can complete the quantitative analysis and confirmation rapidly and accurately. It is necessary to further evaluate and analyze the applicability of quantitative detection methods for genetically modified food to assure their reliability[9-12]. In the EU, corresponded by Bundesinstitut fuer gesundheitlichen Verbraucherschutz und Veterinaermedizin (BgVV, Berlin, Germany), an inter-laboratory study using 3 different types of real-time PCR to analyze genetically modified food quality has been done to evaluate the detection methods using genetically modified soybean *RRS*[13]. Japanese scientists developed methods using reference molecules in stead of reference material to solve the difficulty of obtaining seeds from genetically modified crops as certified reference material for detection[14]. For the requirement of quantitative analysis and confirmation, an inter-laboratory study has also been done. The main purpose includes identifying the limit of quantitative detection, evaluating the credibility of quantitative analysis and the applicability of different instruments, and assuring the applicability of molecular certified reference material and the ability to detect a legally acceptable limit of 5%[15]. Because it is difficult to acquire the certified reference material and the commercialized certified reference material is expensive, the goal of this study is to develop novel reference molecules for laboratories which will need them.

MATERIALS AND METHODS

I. *Chemicals and Materials*

Chloroform and isopropanol were purchased from Merck (Darmstadt, Germany). Hexadecyltrimethyl-ammoniumbromide (CTAB) was purchased from Sigma (St. Louis, MO, USA). Agarose was purchased from Amresco (Solon, Ohio, USA). The immuno-detection kit, produced by SDI (Strategic Diagnostic Inc, Newark, DE, USA), is based on the detection principle of ELISA (enzyme-linked immunosorbent assay). The monoclonal antibody, fixed in the hole of an ELISA plate, against herbicide tolerance CP4EPSPS protein of *RRS* (Roundup Ready™ soybean, Monsanto, MO, USA) and HRP (horseradish-peroxidase) conjugated polyclonal antibody are the basic components of the detection system. 3, 3', 5, 5'-tetramethylbenzidine (TMB) is the substrate of HRP. The technical instructions of the kit are followed to carry out the sample preparation and measurement of the detection method. The detection strips were also produced by SDI (http://www.sdix.com/). Following the instruction, reagents and then a detection strip were added to ground soybeans. The results were determined based on the color of the reaction line after 5 to 20 min.

II. *Preparation of Genetically Modified Soybean Reference Material and Soymilk*

Roundup Ready™ (Monsanto, USA) certified reference materials (CRM) of genetically modified soybeans were 0%, 1%, 2%, and 5% (w/w) produced by Institute for Reference Materials and Measurements (IRMM, Fluka, Switzerland). Market soybeans were identified as GM or non-GM soybeans through sprouting tests. Soymilk preparation method: adequate soybeans were washed and soaked with water over the top for 4 to 6 hr, and then the soybeans were ground to crude soymilk solution in a Golden Pineapple™ food processor followed by filtration with a fine cloth. The total amount of water added was 9 times of the amount of beans. Various samples were taken at different temperatures after heating when cooling down to room temperature, extracted the DNA from the samples.

III. *Instruments*

The instruments used in this study are real-time PCR Lightcycler system (Roche, Germany), ABI 9700 PCR reactor (USA), and microtiter plate photometer (MicroStation, Kebo Biomed, Spånga, Sweden).

IV. *PCR Primers and Reagents*

The primers and nucleotide probes, listed in Table 1, were synthesized by TIB Molbiol (Berlin, Germany)[13].

V. *Preparation and Purification of DNA*

DNA was extracted and purified based on the CTAB method published by Lipp and colleagues in 1999. About 100 mg of sample was extracted and precipitated with CTAB reagents and then purified with chloroform followed by another precipitation with isopropanol[16].

VI. *PCR Reaction and Analysis*

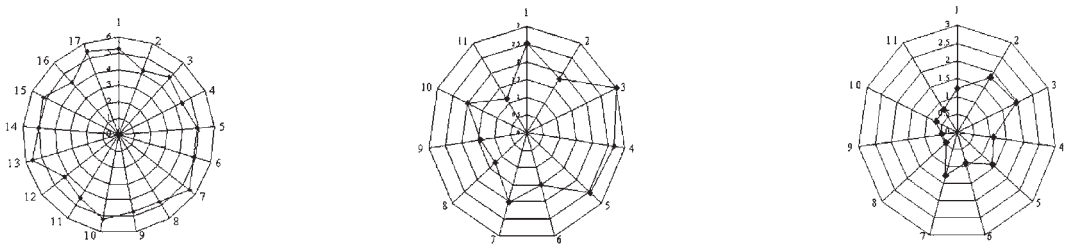
PCR reagents are deoxynucleoside triphosphate

Table 1. Primers and probes used in this study [13]

Primer (Probe)	
Target *RRS* gene[a]	
RR1-F, 10 μmol/L	5'—CATTTGGAGAGGACACGCTGA—3'
RR1-R, 10 μmol/L	5'—GAGCCATGTTGTTAATTTGTGCC—3'
RR1, 10 μmol/L	5'—(FAM)—CAAGCTGACTCTAGCAGATCTTTC-(TAMRA)—3'
Target *lectin* gene[b]	
GM1-F, 10 μmol/L	5'—CCAGCTTCGCCGCTTCCTTC—3'
GM1-R, 10 μmol/L	5'—GAAGGCAAGCCCATCTGCAAGCC—3'
GM1, 10 μmol/L	5'—(FAM)—CTTCACCTTCTATGCCCCTGACAC— (TAMRA) — 3'

[a]The primer pair RR1-F/RR1-R together with the probe RR1 is specific for the detection of the genetic modification in Roundup Ready™ soybeans from Monsanto and should be used for the RR soya system. The PCR product is 74 bp in length.

[b]The primer pair GM1-F/GM1-R together with the probe GM1 is specific for the detection of the single copy *lectin* gene in the soya genome (*Glycine max* L.) and should be used for the soya reference system. The PCR product is 74 bp in length.



(A) (B) (C)

Figure 1. There are 17, 11 and 11 independent tests for genetically modified soybeans (*RRS*) for 5% (part A), 2% (part B) and 1% (part C) reference materials. Mean ± SD values are 4.89 ± 0.45, 1.98 ± 0.63 and 1.09 ± 0.05, respectively.

(dNTP) solution, containing 2.5 mmol/L each of dATP, dCTP, dGTP, and dUTP (20 mmol/L), buffer solution (in 10-fold concentration) without $MgCl_2$, containing the passive, reference dye, $MgCl_2$-solution (c = 25 mmol/L), and LightCycler FastStart DNA master hybprobe (Roche Diagnostics GmbH, Mannheim, Germany).

PCR conditions on the LightCycler follow the instruction of the LightCycler. At least one control group (e.g. 2% of *RRS*) and one negative control group (sterile water) were used in each PCR-run. RR-Soya and Soya total volume of 18.0 μL reference mastermix contain 3.6 μL of $MgCl_2$-solution (25 mM) and 14.4 μL of RR-Soya or Soya-reference mastermix. After adding 2 μL of DNA sample, the reaction mix becomes 20 μL total. PCR program is: denaturation at 95°C for 60 sec, 45 cycles of amplification at 95°C for 5 sec and at 60°C for 25 sec, and finally cooling at 30°C for 45 sec.

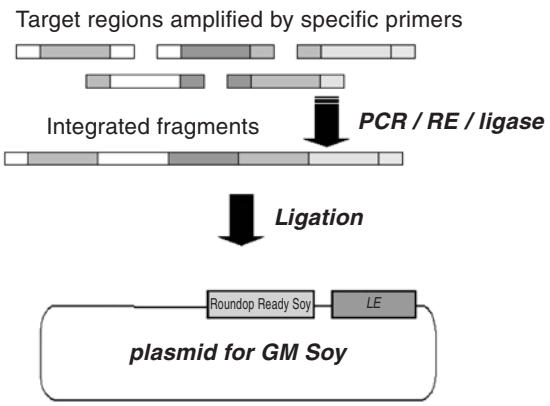


Figure 2. Diagram of pSAM2 derivative from TOPOPCRII™ plasmid.

RESULTS AND DISCUSSION

I. *Detection Tests of Genetically Modified Soybean Using Real-time PCR and Molecular Reference Materials*

The detection of genetically modified soybean (Roundup Ready Soybeans, *RRS*) using LightCycler real-time PCR system was studied in this research. Detection tests of 5%, 2%, and 1% reference materials of genetically modified soybean using real-time PCR were repeated 17, 11, and 11 times, respectively. Results (Mean ± SD), shown in Figure 1, are 4.89 ± 2.17, 1.98 ± 1.2, and 1.09 ± 0.48, respectively. The same real-time PCR method was applied to 28 samples of soybean from the market.

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Table 2. Data analysis of GM soy by different materials in house testing

	Sample 1[a]		Sample 2[a]		Sample 3[a]	
Reference material	CRM[b]	pSAM2	CRM[b]	pSAM2	CRM[b]	pSAM2
Repeat test number	6	6	6	6	6	6
Mean	5.45	4.83	2.42	2.09	1.2	1.08
SD	0.315	0.448	0.133	0.121	0.089	0.095

[a]Sample 1, Sample 2 and Sample 3 were Certified Reference Material GM-soybeans content 5, 2 and 1%, respectively.

[b]Certified reference materials.

Results showed that all of them are genetically modified soybeans. In detail, 21% (6/28) of them contained 10 to 20%, 58% (16/28) contained 20 to 30%, and 21% (6/28) contained 30 to 50% of genetically modified soybeans. As to processed soybeans, 5 samples (5/14) were non-GMO products, 4 samples (4/14) contained 20 to 50%, and 5 samples (5/14) contained more than 50% of genetically modified soybeans. Compared with previous (2001) detection tests of 5%, 2%, and 1% reference materials of genetically modified soybean repeated 25, 15, and 14 times, results (Mean ± SD) were 5.09 ± 7.61, 2.22 ± 2.01, and 1.19 ± 0.70, respectively. Obviously, it is improved than the former testing. Although the reaction system, enzyme system, primers, and probes are the same, but reaction conditions were adjusted in this study. For example, the concentrations of the primers were lowered, PCR extension time was prolonged and double distilled water was replaced with TE (Tris-EDTA) buffer as the solvent. It was found that the reproductivity and stability are thus improved.

Because it is difficult to acquire the certified reference material, and to prepare the standardized concentrations precisely, molecular reference materials for long term application were studied in this research. Various fragments of *lectin* genes were linked with *RRS* genes using ligase, and then they were inserted into the plasmid TOPOPCRII^{TM} (Figure 2) using TOPO TA Cloning kit (Invitrogen^{TM}). This plasmid, called pSAM2, was transferred into host *Escherichia coli* for reproduction, and then was extracted and purified followed by determination of its exact concentration. Data analysis of GM soybean in different samples and reference materials in house testing showed that the detection results of using CRM (certificated reference material by IRMM, Fluka, Switzerland) and pSAM2 molecular reference material to the same sample are similar (Table 2). Results (Sn, mean ± SD) of 6 detections on 3 samples, S1, S2, and S3, using CRM are (S1, 5.45 ± 0.315), (S2, 2.42 ± 0.133), and (S3, 1.20 ± 0.089). As to pSAM2 molecular reference material, results (Sn, mean ± SD) are (S1, 4.83 ± 0.448), (S2, 2.09 ± 0.121), and (S3, 1.08 ± 0.095). Detection results on each one of three samples using CRM and pSAM2 molecular reference material are similar. It is better than the result (25%) from the inter-laboratory studies in the EU on genetically modified foods[13]. In Japan, the results of 10 detections on 5% and 1% of genetically modified soybeans using a novel molecular reference material were 5.4% and 1.1% (mean values), and the highest and the lowest values were 4.9% and 5.8% under 95% confident zone[14,15]. Although this study used a LightCycler PCR instrument instead of an ABI7700 PCR instrument which was used by Japanese scientists, the results are similar. Therefore, this is a practicable strategy and method using pSAM2 molecular reference material to detect genetically modified soybeans (Table 3).

Table 3. General properties of reference materials and reference molecules

	Reference materials	Reference molecules
Resource	Limited	Unlimited
Genuine seeds	Essentiality	Nonessential
Source	Single standard for each GM trail	Single and multiple standard for each GM trail
Quality control	Difficult	Easy
Bias	Increase by dilution	Decrease by dilution
Homogenization & calibration	Difficult	High & easy
Price	High	Higher

II. *Detection of the Change of Genetically Modified Proteins and Cloned Genes during Heating Process in Self-prepared Soymilk Using ELISA, PCR, and Real-time PCR Methods*

ELISA, PCR, and real-time PCR methods were used to detect the change of genetically modified proteins and cloned genes during the heating process in self-prepared soymilk. ELISA was used to detect the change of genetically modified proteins during heating process in self-prepared soymilk. The value of OD_{450} started to decrease after being heated at 70v for 1 minute. OD_{450} was lower than half of original value after being heated for 3 to 9 min. OD_{450} came to the lowest value after being heated at 75°C for 1 to 3 min meaning that *RRS* specific proteins can not be accurately detected. Therefore, genetically modified proteins in soymilk which has been heated over 70°C are denatured rapidly, making detection exceptionally difficult. PCR method was useful to detect

genetically modified genes. Under common conditions (heated toward 50 to 100°C boiling), 35S promoter, NOS terminator, and CP4EPSPS of *RRS* genes can be detected. Results became negative after heated at 121°C for 10, 20, and 30 min meaning target genes were affected by heating as well. Most of the target genes were degraded at 100°C, but they still could be extracted and detected by the real-time PCR method. DNA was degraded into much smaller fragments after heated at 121°C for more than 10 min, so that it is difficult to be extracted and detected. If shorter target genes for PCR detection are used, the effect of DNA degradation from heating can be decreased. The length of PCR products from genetically modified soybean for detection and primer pair of original control gene used for the PCR method in this study is only 74 bp, which is much smaller than other PCR methods. Therefore, the effect from heating is decreased tremendously.

III. *Problems and Solutions of the Detection*

(I) Improvement of limit of detection (LOD): The quantitative analysis using molecular amplification methods has been widely applied to research in laboratories, including pharmacological genomics and molecular detections. One category is called target amplification methods, including polymerase chain reaction (PCR), strand displacement amplification (SDA), ligase chain reaction (LCR), and nucleic acid sequence-based amplification (NASBA). They are very sensitive, and they can be coupled with fluorescence detection, luminescence detection, or colloidal analysis[2,17,18]. Another category is called signal amplification technology, including branched DNA (bDNA), hybrid capture and cleavase, X-ray fluorescence spectrometry[19,20], and methods using substitute markers to detect target nucleotides. Rolling circle amplification (RCA) is a novel method using the signal amplification technology. In addition, the most important improvement on PCR is to detect products simultaneously in one sealed tube using fluorescence[21,22]. Not only does it become more sensitive and specific, but it can also detect multiple targets quantitatively at the same time. To control differences among various samples, certain amount of reference gene is added to each sample so that the specificity of TaqMan^{TM} reaction can be differentiated from different crops effectively. Meanwhile the Ct (crossing point) values of different samples are normalized by consistent reference gene. The content in percentage is calculated directly from the ratio of cloned gene and endogenous gene in the tube. LOD can be very different among various detection systems due to conditions of purification, design of primers and probes, and recipe of reagents. LOD of detecting CRM 1%, 2%, and 5% of genetically modified soybean (*RRS*) was studied in this research. The LOD results of detecting the house keeping gene *lectin* are 1.25, 0.66, 0.69, and 0.68 ng/μL, respectively. As to the cloned *RRS* gene, the LOD results are 2.5, 1.31, 1.37, and 2.69 ng/μL, respectively (Figure 3, A to F). It showed that LOD of detecting *lectin* gene is lower than *RRS* gene at the same concentration meaning that the detection sensitivity for *lectin* is better than *RRS*. When using reference molecules pSAM2, the LOD results of detecting *lectin* and *RRS* gene are 1.90×10^2 and 2.1×10^2 copies, respectively (Table 4). These results are similar to the results using CRM. Comparing the results using certified reference material CRM *RRS* with using reference molecules pSAM2, the LOD results of the detecting *lectin* gene are between 2.43×10^2 and 4.63×10^2 copies, which is a higher range than that for LOD (1.90×10^2 copies) when using reference molecules pSAM2. As for the *RRS* gene, the LOD results are between 4.86×10^2 and 9.95×10^2 copies, which is also a higher range than that for LOD (2.1×10^2 copies) when using reference molecules pSAM2. It showed that the LOD results of using reference molecules pSAM2 are better than CRM *RRS*. In addition, it is much easier to acquire and prepare the reference molecules pSAM2. These results indicated that pSAM2 is good for routine application.

Table 4. Comparison of the limit of detection in different RRS reference materials

Reference materials	Target gene	LOD (copies numbers)
CRM 1%		4.63×10^2
CRM 2%		2.44×10^2
CRM 5%	***Lectin***	2.56×10^2
pSAM2		1.90×10^2
CRM 1%		9.26×10^2
CRM 2%		4.85×10^2
CRM 5%	***RRS***	5.07×10^2
pSAM2		2.10×10^2

(II) Difficulties of detecting degraded DNA: Scientists have found it difficult to detect degraded DNA in processed food when studying genetically modified food[23,24]. This research further studied how DNA degradation affects detection results. Real-time PCR was used to detect the extent of DNA degradation after heating soymilk to various temperatures (Figure 4). Samples were taken from soymilk and 10-fold concentrated soymilk at different temperatures from 40 to 100°C, after boiling for 30 min, at 121°C for 15, 25, and 35 min followed by DNA extraction. The Ct values of *RRS* gene and *lectin* gene are similar (Figure 4 A). The ΔCt *RRS*/Le values (Ct_{RRS}/Ct_{lectin}) of market soymilk are between 1.06 and 1.07 while averaging 1.07. The ΔCt RRS/Le_{10X} values ($Ct_{RRS10X}/Ct_{lectin10X}$)(10-fold concentrated soymilk) are between 1.06 and 1.08 while averaging 1.07 (Figure 4 B). It showed that the degradation extent of cloned gene and reference gene in soymilk is consistent regardless of the concentration and the temperature during processing, so that the calculated result will not be affected. This is similar to the result by Pan *et al.*[23], who detected 5% of genetically modified soybean in self-made fermented food such as fermented

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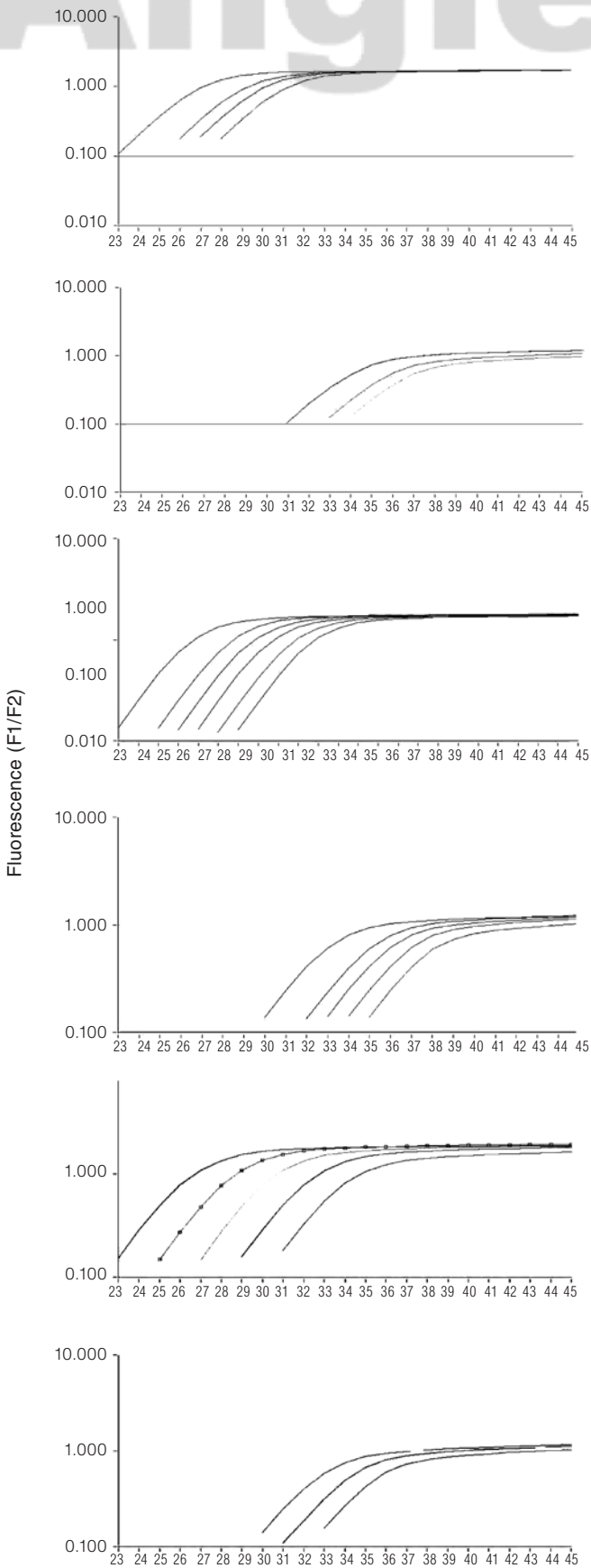

CRM 1% RRS	Lectin
Slope	-2.977
Intercept	30.85
Error	0.02
R	-1.00
LOD	1.25

A

CRM 1% RRS	RRS
Slope	-3.428
Intercept	29.79
Error	0.435
R	-1.00
LOD	2.5

B

CRM 2% RRS	Lectin
Slope	-3.366
Intercept	28.28
Error	0.0137
R	-1.00
LOD	0.66

C

CRM 2% RRS	RRS
Slope	-3.298
Intercept	29.44
Error	0.018
R	-1.00
LOD	1.31

D

CRM 5% RRS	Lectin
Slope	-3.200
Intercept	28.82
Error	0.0440
R	-1.00
LOD	0.69

E

CRM 5% RRS	RRS
Slope	-2.541
Intercept	29.49
Error	0.0554
R	-1.00
LOD	1.37

F

Figure 3. Detection limit test results of *lectin* and *RRS* genes for CRM 1, 2 & 5% GM soybean (*RRS*). Standard curve analysis in right boxes and different concentrations reaction in left plots.

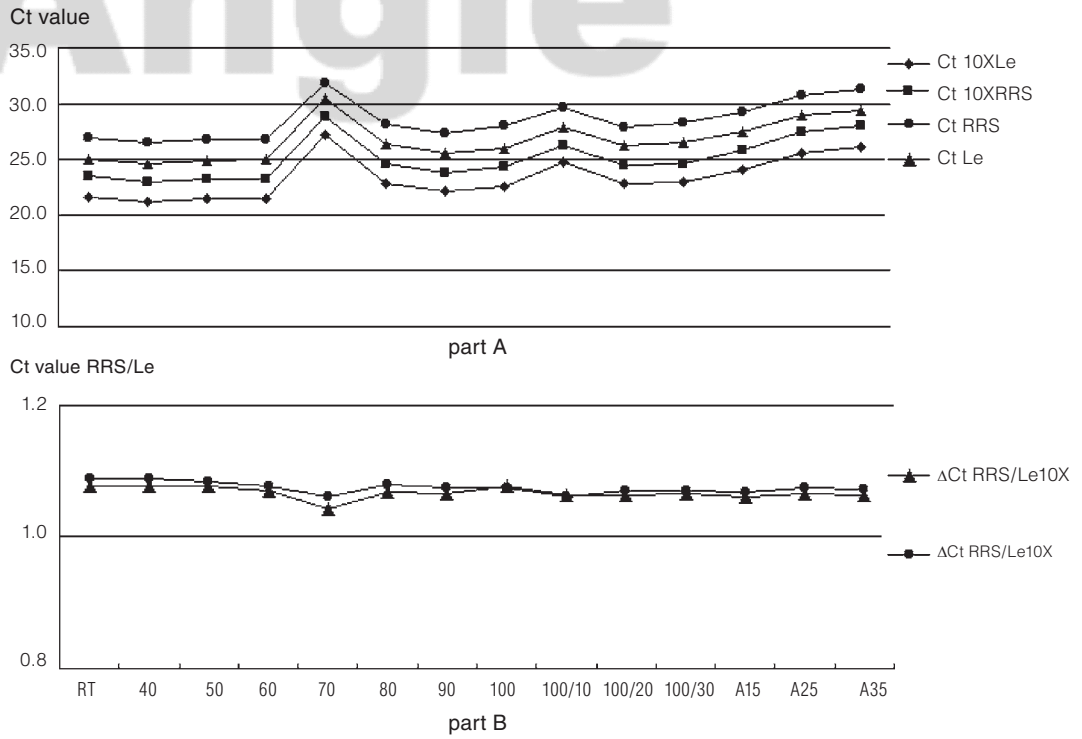

Figure 4. Ct and ΔCt value analysis during soymilk heat treatment in–house processing. Part A & B were Ct & ΔCt during heat treatment. Y axial scales represent the sampling points for the heat treatment from the room temperature (RT)to 100°C, add 10, 20 and 30 min (100/10, 100/20 and 100/30), and autoclaved with 15, 25 and 35 min (A15, A25 and A35), respectively. The ΔCt *RRS*/Le values was Ct_{RRS}/Ct_{lectin} and the ΔCt *RRS*/Le10X values was $Ct_{RRS10X}/Ct_{lectin10X}$.

tofu. It is 5 % at the measurable period. Target DNA still can be detected after 120 to 150 days. It is also similar to the result by Torsten *et al*.[24] who used various primers to detect the DNA in processed food. The extent of DNA degradation is not the same. The content of genetically modified components is the same in crude material and in processed product such as tofu and soymilk. However, both Pan *et al*. and Torsten *et al*.[23,24] did not study the relationship between the extent of DNA degradation and detection results. With previous studies and this research, the reliability of detecting genetically modified soybean and its processed product is confirmed. However, PCR product in this research is 70 to 80 bp, which is much smaller than the product in the study by Pan *et al*. (100 to 200 bp) and by Torsten *et al*. (500~600 bp). It is concluded that the detection result for processed products will be better when the PCR product is smaller.

IV. *Applicability*

Department of Health of the Executive Yuan in Taiwan has announced the Guidelines for safety assessment of genetically modified foods[25]. Because of increasing international trade, the allowable content and other regulations of every country related to the genetically modified food must be considered[26]. Most researches focus on the detection techniques and methods development[27]. Studying and developing reference materials have been very few, especially with regard to molecules reference materials. In the case of quantitative detection, the content of target material is calculated based on a standard regression curve which is made from content-known certified reference materials. In previous research, certified reference materials were prepared by a specific laboratory and then provided or sold to various laboratories. It is difficult to acquire, maintain, and calibrate the certified reference materials, and commercialized molecular certified reference materials are expensive (Table 3). However, the novel reference molecules developed in this research can be prepared, reproduced, and calibrated by common molecular laboratories. The results in this research can also be used by various organizations as references. The intra-laboratory tests on the novel reference molecules developed in this research have been completed. To fulfill the requirement and reliability of local and international regulations, it is necessary to conduct further inter-laboratory tests so that the applicability of quantitative detection methods for genetically modified food will be well analyzed and evaluated[15,28,29,30].

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