Food Sci. Biotechnol. 25(4): 1-7 (2016)
DOI 10.1007/s10068-016-0

Food Science
and
Biotechnology

Rapid Screening of Roundup Ready Soybean in Food Samples by a Hand-held PCR Device

Hsiang-Yun Tung[1], Sue-Hong Wang[2], Yu-Cheng Chiang[3], and Ming-Shiun Tsai[1,4,*]

[1]*College of Biotechnology and Bioresources, Da-Yeh University, Changhua 51591, Taiwan, China*
[2]*Department of Biomedical Sciences, Chung Shan Medical University, Taichung 402, Taiwan, China*
[3]*Department of Food Science and Technology, Hung Kuang University, Taichung 43302, Taiwan, China*
[4]*Department of BioIndustry Technology, Da-Yeh University, Changhua 51591, Taiwan, China*

Received December 26, 2015
Revised April 10, 2016
Accepted May 18, 2016
Published online August 31, 2016

*Corresponding Author
Tel: +886-4-8511888 (ext 2300)
Fax: +886-4-8511320
E-mail: tsaims1@mail.dyu.edu.tw

pISSN 1226-7708
eISSN 2092-6456

Abstract Insulated isothermal PCR (iiPCR) method was recently available for rapid on-site detection of roundup ready soybean (RRS; event GTS40-3-2) in food materials and products. Performance of this method was evaluated in this study. The 100% detection endpoint for the RRS by iiPCR was found in samples containing 0.1% RRS, equivalent to the results of the reference real-time PCR (rtPCR). Analysis of nucleic acids of soybean-based processed food products indicated 95% agreement between the iiPCR and rtPCR for RRS detection. By testing soybean milk and tofu samples using simple pretreatment methods, we found that the agreements between iiPCR and rtPCR methods of the aforementioned samples were 80% and 90%, respectively. Replicated tests of all discrepant samples implied that these samples had trace amounts of RRS, suggesting that the iiPCR system is more sensitive than the rtPCR method. In conclusion, the iiPCR technology can be a useful point-of-need tool to help make a timely decision in the consumption of genetically modified organisms.

Keywords: genetically modified organisms, insulated isothermal PCR technology, manual DNA extraction cartridge, on-site detection, roundup ready soybean

Introduction

Genetic modification of economic crops is a promising strategy for versatile purposes (1,2). Various crops have been genetically modified (GM) for the benefits of food production efficiency (e.g., insecticide-/herbicide-resistant soybean and cotton) and nutritional contents (GM rice with increased levels of iron and vitamins). The genetically modified organisms (GMOs) have been grown worldwide with a recorded 181.5 million hectares in 28 countries in 2014, with the USA being the largest GMO producer in 2013/2014 (2,3). Among the GM soybean events, the roundup ready soybean (RRS; event GTS40-3-2) is the most approved GMO worldwide, produced mainly in the USA, Argentina, and Brazil (2,4).

The public in general have concerns over the potential health and ecological risks of GMOs and their products (1,5-7). To help consumers make an informed decision about consumption of GMOs, more than 60 countries have implemented labeling requirement of GMOs and GMO-derived foods/feeds with different threshold levels allowed, e.g., 0.9% in the European Union, 3% in Taiwan and Korea, 5% in Japan, and 0% in China (5,8-11). Furthermore, the labeling of non-GMO ingredients and products has been initiated by the non-GMO project (http://www.nongmoproject.org/) (5). To regulate the marketing of GMO products, history traceability has also been implemented in areas such as the European Union (1,12).

In GMOs, one or more specific exogenous sequences are inserted into the plant genome through DNA recombination event to express target protein(s). Protein- and DNA-based tests have been utilized to monitor the usage of GMOs in raw ingredients and foods (1). Because of the higher stability of DNA than proteins and the high sensitivity and specificity of polymerase chain reaction (PCR), conventional PCR and real-time PCR (rtPCR) assays have become the most accepted tools for GMO monitoring (1,11,13-16). Screening-, gene-, construct- and event-specific markers have been targeted for different purposes in GMO monitoring (17).

On-site detection of GMOs could facilitate manufacturers and consumers to make timely informed decisions and help them to decrease the cost of GMO management by reducing the number of specimen. However, applications of conventional and/or rtPCR assays requiring a specialized laboratory, technician, and instrument have been restricted mostly to the central laboratories. A pyrosequencing-based portable sequencer (10) and the loop-mediated isothermal amplification (LAMP) (18,19) have been developed for on-site GMO detections. Because of complicated DNA extraction, PCR amplification, and single-strand DNA preparation, the pyrosequencing method

needs further development(s).

By searching for commercial PCR-based tests for GMO markers, we found that the POCKIT™ GMO Soy RRS Reagent Set (GeneReach, Taichung, Taiwan) was available recently for on-site GMO monitoring. Based on the TaqMan probe hydrolysis-insulated isothermal PCR (iiPCR) methodology (20,21), this set was provided in a lyophilized format to allow easy shipping, long-term storage and worked on a field-deployable device (POCKIT™ Nucleic Acid Analyzer and POCKIT™; GeneReach). Furthermore, the iiPCR/POCKIT™ system has been successfully applied to detect various viral and bacterial pathogens in animals with high sensitivities and specificities (20,22-30). Without any post-amplification step, this system drastically reduces the false positive risks due to carry-over contaminations. A nucleic acid extraction step is generally needed before PCR amplification. Most commonly used DNA extraction methods or commercial kits require laboratory instruments and are time consuming. We also found a rapid and easy-to-use manual DNA extraction cartridge: the Grind-N-Go DNA Extraction Set (GeneReach), which was available recently to facilitate quick on-site nucleic acid extraction. Together, the Grind-N-Go DNA Extraction Set and the iiPCR/POCKIT™ system should enable easy and rapid on-site GMO detections.

In this study, the specificity, sensitivity, and repeatability of the RRS iiPCR/POCKIT™ system (POCKIT™ GMO Soy RRS Reagent Set) and its combination with the manual extraction cartridge (Grind-N-Go DNA Extraction Set) for RRS detections in soybean-based materials and food products were evaluated.

Materials and Methods

Samples Certified reference materials (CRMs) including genomic DNAs of events A2704-12 and A5547-127 and homogenized seed powders of non-GM soybean and events GTS40-3-2, DP-356043, Mon87705, and Mon87708 were purchased from the European Union Joint Research Center (JRC-IRMM; JRC, Geel, Belgium). Seed samples including *Vigna angularis*, *V. radiata*, *Zea mays*, *Arachis hypogaea*, and *Oryza sativa* were purchased from local markets in Taiwan. The soybean-based foods tested, including soybean milk, tofu, pressed tofu (tofu of low water content), soft tofu (tofu of high water content), fried tofu, fried tofu skin (tofu skin is the film formed on the liquid surface during the boiling of soybean milk), and vegetarian ham were purchased from local markets. The pGH-based control plasmid (http://dnasu.org/DNASU/GetVectorDetail.do?vectorid=499) was synthesized to contain an RRS-specific fragment (nt127 to 276, GenBank accession number AB180968.1) (MDBio, Taipei, Taiwan) as a positive control for rtPCR and iiPCR tests.

DNA extraction Embryos from overnight-soaked seeds or 50 mg of seed powder were used for nucleic acid extractions. To prepare DNA of known quantity, the dodecyl trimethyl ammonium bromide (DTAB)-acetyl trimethyl ammonium bromide (CTAB) method or taco™ mini method was performed. The DTAB-CTAB method was performed using the DTAB-CTAB DNA Extraction Kit (GeneReach). The samples were homogenized in 600-μL DTAB solution, incubated for 5 min at 75°C, and cooled to room temperature. The mixture was mixed with 700 μL chloroform for 20 s and centrifuged at 12,000x *g* for 5 min. For RNase treatment, supernatant was treated with 10 μg/mL RNase A (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37°C, mixed with equal volume of chloroform, and centrifuged at 12,000x g for 5 min. Subsequently, 200 μL supernatant was mixed with 10% (v/v) CTAB solution, incubated at 75°C for 5 min, cooled to room temperature, and centrifuged at 12,000x *g* for 10 min. The supernatant was mixed with 150 μL dissolving solution, incubated at 75°C for 5 min, and centrifuged for 5 min at 12,000x *g*. The aqueous phase was mixed with 300 μL of 95% (v/v) ethanol and centrifuged at 12,000x *g* at 4°C for 5 min. The pellet was washed with 200 μL of 75% (v/v) ethanol, dried at room temperature, and dissolved in 100 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Extraction using the taco™ mini Nucleic Acid Automatic Extraction System (taco™ mini; GeneReach) was performed by following the manufacturer's instructions. In brief, the wells of the extraction plate were filled with the designated reagent buffers. The samples were homogenized in 400-μL lysis buffer and centrifuged at 12,000x *g* for 5 min. After the supernatant was added into the sample well, the plate was loaded into a taco mini for automatic DNA extraction. Nucleic acid extracts eluted in 50-μL elution buffer were collected individually and stored at −80°C for later use. The concentrations of nucleic acids were determined by a UV spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA) ($OD_{260\ nm}$/$OD_{280\ nm}$ =1.6–2.0) and stored at −20°C until it was used.

The manual DNA extraction cartridge (Grind-N-Go; GeneReach), a NaOH-based manual extraction method, is worked in an extraction cartridge containing two buffers in two separate wells. After the sample was loaded into the sample well, the grinder was locked into the sample well. Subsequently, the sample well/grinder unit was detached and placed into the lysis buffer well. Homogenization of the sample was achieved by twisting the grinder back and forth at least 10 times. Finally, DNA extraction was completed by shaking the cartridge a few times to mix the homogenate with the neutralization buffer in the outside well. The extract was transferred to a downstream iiPCR reaction with an inoculating loop (approximately 2 μL) for RRS detection.

Real-time PCR (rtPCR) The rtPCR for RRS detection published by Taiwan Food and Drug Administration (15) was used as the reference method in this study. The primers and probes targeted specifically to the junction regions spanning the soybean host genome and the *35S* promoter, a combination found only in the soybean GTS40-3-2 event. They were the forward primer (5'-GCCTTTCCTTTATCGCAATGATG-3'), reverse primer (5'-TCAAAATAAGATCATACATACAGGTTAAAATAAAC-3'), and probe (FAM-5'-CCACCTTCCTTTTCCATTTGGGTTCCC-3'-TAMRA). The 25-μL reaction mixture contained 1x TaqMan Universal Master

Mix (BioMi, Taichung, Taiwan), 0.25 μM forward primer, 0.25 μM reverse primer, 0.22 μM probe, 2 unit Taq DNA polymerase (BioMi), and DNA sample. The rtPCR was performed on an Applied Biosystems® Step One Plus™ system (Thermo Fisher Scientific) with the following conditions: denaturation at 93°C for 5 min, followed by 45 cycles of 93°C for 15 s and 60°C for 1 min. Tests of serial dilutions of the control plasmid in a typical test showed that the linearity range was between 10^6 to 10 copies with a y-intercept of 38.7 and a slope of −3.35, indicating an amplification efficiency of 98.7%. The coefficient of determination (r^2) was 0.99. Based on the replicate analysis of 100 ng DNA of 0.1% of RRS CRM, a cutoff threshold cycle (Ct) was assigned: the sample with a Ct≤36 was considered as RRS positive (>0.1% RRS), while the sample with a Ct>36 was considered as RRS negative.

RRS iiPCR assay The RRS iiPCR test (POCKIT™ GMO Soy RRS Reagent Set; GeneReach) was provided in a lyophilized format. To perform the test, the premix buffer was first transferred with a dropper provided in the kit to reconstitute the RRS iiPCR reagent, which was subsequently mixed with a loopful of the nucleic acid extract of the test sample with a loop provided. Subsequently, the final mixture was transferred with a fresh dropper to an R-tube™ (GeneReach), which was subsequently placed into the POCKIT™ Micro device to complete the reaction. Based on the default thresholds, "+", "–", and "?" were shown on the display screen for positive, negative, and suspect results, respectively, in approximately 30 min. A "?" result indicated that the signals were ambiguous and the sample should be tested again.

Statistical analysis For the performance agreement between two test methods, the Kappa index was calculated from a 2×2 contingency table by using SPSS v. 14 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

The GTS40-3-2 (RRS) event contains exogenous sequences encoding the CaMV *35S* promoter, the chloroplast transit peptide (*CP4-epsps*), and the *nos* terminator. Assays that amplify an event-specific marker rely on primers to amplify the unique junction regions between the transgenic expression cassette sequences and the host genome. The RRS event-specific iiPCR system (POCKIT™ GMO Soy RRS Reagent Set; GeneReach) targeted the *35S* promoter element and its flanking host genome. In this study, the performance of this reagent set on the hand-held POCKIT™ Micro device for the detection of the RRS element was evaluated.

RRS iiPCR Reagent Set specifically detected RRS Events GTS40-3-2 (RRS), A2704-12, A5547-127, DP-356043, Mon87705, and Mon87708 have been approved for human consumption in Taiwan (3). Among these, events A2704-12 and A5547-127 contained the CaMV *35S* promoter element and both Mon87705 and Mon87708 had the *CP4-epsps* gene. To test the analytical specificity of the RRS iiPCR Reagent Set, 100 ng of genomic DNA was prepared from CRMs of these events and a non-GM soybean by the DTAB-CTAB extraction method and then analyzed in triplicate. Negative results with the non-GM soybean indicated that the reagent set did not cross-react with any endogenous sequences of wild-type soybean. Positive RRS iiPCR results were obtained only with the GTS40-3-2 (RRS) sample, demonstrating that the RRS iiPCR Reagent Set could specifically detect the GTS40-3-2 event. Furthermore, seeds of other crops, including *Vigna angularis* (adzuki bean), *V. radiata* (mung bean), *Z. mays* (corn), *A. hypogaea* (peanut), and *O. sativa* (rice), have been commonly mixed with soybean in processed foods in Taiwan. When 100 ng DNA samples prepared from embryos of these species were analyzed by the reagent set, all were found to generate negative results, indicating that the RRS iiPCR Reagent Set did not cross-react with DNAs of these species. These results suggested that the RRS iiPCR Reagent Set could be used to monitor RRS in processed foods made with mixed ingredients.

Detection limit of RRS iiPCR Reagent Set To determine the sensitivity of the RRS iiPCR Reagent Set, 100 ng genomic DNA containing 10, 1, 0.1, and 0.01% (w/w) of GTS40-3-2 (RRS) DNA was prepared by diluting RRS genomic DNA serially with non-GM soybean genomic DNA and then analyzed in 20 replicates. The RRS iiPCR Reagent Set detected 20/20 (100%) of the 10, 1, and 0.1% RRS, 7/20 (35%) of the 0.01% RRS, and none of the negative control samples. Limit of detection is the lowest amount or concentration of an analyte that can be detected reliably and ensures ≤5% false negative results (31). Thus, the 100% detection endpoint of the RRS iiPCR Reagent Set is observed to be the 0.1% RRS, indicating a high level of sensitivity. The detection limit of RRS iiPCR Reagent Set is comparable to the reference rtPCR, the padlock probe ligation in combination with microarray detection (32), and the multiplex PCR methods (33,34). Therefore, for countries implementing labeling requirement of GMOs with different threshold levels, such as 0.9% in the European Union and 3% in Taiwan and Korea, the RRS iiPCR Reagent Set could be a useful tool for RRS screening.

Intra- and inter-assay repeatabilities of RRS iiPCR Reagent Set The repeatability of the RRS iiPCR Reagent Set was assessed by analyzing samples containing target RRS DNA at 1 (0.1%) and 10 folds (1.0%) of the assay's detection endpoint from the detection limit described above. Six replicates of each concentration were tested in three independent runs to evaluate the intra- and inter-assay repeatabilities. All of the 36 reactions showed positive results, indicating that the RRS iiPCR Reagent Set could provide consistent RRS detection results.

Detection of RRS DNAs from different soybean-based foods by RRS iiPCR Reagent Set: Comparison with RRS rtPCR The RRS iiPCR Reagent Set was evaluated further for RRS detections in soybean-

Table 1. Detection of RRS DNAs in soybean-based processed foods by RRS rtPCR method and RRS iiPCR Reagent Set

Sample	No.	rtPCR[1)]		iiPCR
		Ct	Result	
Tofu	1	32.02	+	+
	2	ND	-	-
	3	ND	-	-
	4	31.81	+	+
	5	31.54	+	+
	6	ND	-	-
	7	ND	-	-
	8	ND	-	-
	9	ND	-	-
	10	ND	-	-
	11	ND	-	-
Pressed tofu	1	31.81	+	+
	2	32.65	+	+
	3	ND	-	-
	4	ND	-	-
	5	33.37	+	+
	6	33.40	+	+
	7	ND	-	-
	8	ND	-	-
Soft tofu	1	ND	-	-
	2	ND	-	-
Vegetarian ham	1	31.02	+	+
	2	ND	-	-
	3	37.19	-	+
	4	ND	-	-
	5	38.22	-	-
	6	ND	-	-
Fried tofu	1	35.13	+	+
	2	ND	-	-
	3	29.26	+	+
	4	30.41	+	+
	5	ND	-	-
Fried tofu skin	1	ND	-	-
	2	ND	-	+
	3	ND	-	-
	4	31.27	+	+
	5	31.29	+	+
	6	32.97	+	+
	7	ND	-	-
	8	32.1	+	+

[1)]Ct>36 was considered as a negative result (15); ND, not determined

based products by using the RRS rtPCR as a comparison reference, which had a detection limit at 0.1% (w/w) RRS DNA (15). DNA extracts of 40 commonly consumed soybean-based food products in Taiwan were prepared by the manual DNA extraction cartridge and then tested by the iiPCR and the rtPCR in parallel. The soybean-based food products were tofu, pressed tofu, soft tofu, fried tofu, fried tofu skin, and vegetarian ham. Totally, 15 samples were positive and 25 were negative by the RRS rtPCR assays (Table 1). All rtPCR positive samples were also positive by the iiPCR Reagent Set while 2 of the 25 rtPCR negative samples (Ct=37.19 and ND in vegetarian ham and fried tofu skin) were positive by the iiPCR Reagent Set. Analysis of the 2x2 contingency table determined that a 95% agreement (38/40; 95% confidence interval, 81.79-99.13%) was found between these two assays (kappa value=0.90), suggesting that the performance of the RRS iiPCR Reagent Set was in accordance with that of the RRS rtPCR. Therefore, the results preliminarily show that the RRS iiPCR Reagent Set could detect RRS DNA in tofu, pressed tofu, soft tofu, fried tofu, fried tofu skin, and vegetarian ham with performance similar to the reference RRS rtPCR.

Detection of RRS in soybean milk directly by RRS iiPCR Reagent Set Because soybean milk is essentially the aqueous extract of homogenized soybean, it was suggested that soybean milk could be tested directly with a simple dilution step by the RRS iiPCR Reagent Set. To evaluate the feasibility of this protocol, 30 soybean milk samples were collected locally and subjected to the RRS iiPCR Reagent Set and the reference RRS rtPCR. The RRS rtPCR, testing 100 ng DNA, was performed following the published protocol (15). For the RRS iiPCR Reagent Set, soybean milk was diluted 10 folds in distilled water, and then a loopful (approximately 2 μL) of each diluent was added to the reconstituted RRS iiPCR reaction. Among the 30 samples, 13 and 17 samples were positive and negative for the RRS rtPCR tests, respectively (Table 2). All rtPCR positive samples were also positive by the iiPCR Reagent Set; however, 6 samples showed rtPCR negative but iiPCR positive (S2, S4, S13, S19, S23, and S24) (Table 2). Analysis of the 2x2 contingency table (Table 3) determined that the agreement between two methods was 80% (95% confidence interval: 95.15-64.85%; kappa value=0.61). Five of the 6 discrepant samples (S2, S13, S19, S23, and S24) showed Ct values between 36 and 40 (Table 2), suggesting that these samples contained trace amounts of RRS (<0.1%). Subsequently, 5 replicates of all 6 discrepant samples were tested by both methods (Table 4). All samples had at least one test with positive result in the RRS iiPCR Reagent Set and at least one test in the RRS rtPCR with Ct value between 36 and 40 (Table 4), supporting that these 6 samples contained trace amounts of RRS (<0.1%). Therefore, these results imply that the samples suspected of containing trace amounts of RRS (likely around 0.01%) could be detectable by the RRS iiPCR Reagent Set.

Because 30 different soybean milk samples from different commercial sources were directly tested in this examination, the RRS iiPCR Reagent Set could potentially be used for RRS screening in soybean milks produced by different manufacturers and vendors. Therefore, users who needed to define the RRS content in soybean milk samples could submit the RRS iiPCR-positive specimens to a reference laboratory for quantification testing by the RRS rtPCR method.

Detection of RRS in tofu by Grind-N-Go/RRS iiPCR/POCKIT™ Micro system The user-friendly DNA extraction cartridge could be shipped and stored at room temperature. Together, the Grind-N-Go/RRS iiPCR/POCKIT™ Micro system was aimed to facilitate on-site small

Table 2. RRS detections in soybean milks by RRS iiPCR Reagent Set and in tofu-based samples by *Grind-N-Go/RRS iiPCR/POCKIT™ Micro* system: comparisons with RRS rtPCR method

Soybean milk				Tofu			
No.	RRS rtPCR[1)]		iiPCR	No.	RRS rtPCR[1)]		iiPCR
	Ct	Result			Ct	Result	
S1	ND	-	-	T1	ND	-	-
S2	36.08	-	+	T2	ND	-	-
S3	39.37	-	-	T3	38.05	-	-
S4	ND	-	+	T4	ND	-	-
S5	ND	-	-	T5	ND	-	-
S6	ND	-	-	T6	ND	-	-
S7	28.98	+	+	T7	ND	-	-
S8	39.94	-	-	T8	ND	-	-
S9	37.68	-	-	T9	42.59	-	-
S10	29.43	+	+	T10	25.27	+	+
S11	28.46	+	+	T11	27.12	+	+
S12	29.79	+	+	T12	27.20	+	+
S13	39.24	-	+	T13	ND	-	-
S14	28.67	+	+	T14	ND	-	-
S15	28.91	+	+	T15	36.52	-	+
S16	39.03	-	-	T16	ND	-	-
S17	35.39	+	+	T17	38.84	-	+
S18	ND	-	-	T18	38.62	-	+
S19	38.74	-	+	T19	28.58	+	+
S20	ND	-	-	T20	28.48	+	+
S21	ND	-	-	T21	27.36	+	+
S22	29.01	+	+	T22	25.43	+	+
S23	37.02	-	+	T23	40.15	-	-
S24	36.80	-	+	T24	39.01	-	-
S25	29.43	+	+	T25	32.93	+	+
S26	28.55	+	+	T26	34.31	+	+
S27	29.63	+	+	T27	31.06	+	+
S28	ND	-	-	T28	31.22	+	+
S29	28.68	+	+	T29	27.99	+	+
S30	29.06	+	+	T30	28.08	+	+

[1)]Ct > 36 was considered as a negative result (15); ND, not determined

Table 3. Performance comparison between the iiPCR and rtPCR: RRS detections in soybean milk and tofu-based samples

			rtPCR	
			+	-
iiPCR	Soybean milk	+	13	6
		-	0	11
	Tofu	+	13	3
		-	0	14

scale RRS screening for timely and efficiently monitoring and tracing of RRS contamination in raw materials and/or processed foods. Tofu, which was the most consumed soybean-derived foods and the base for other commonly consumed products such as dried tofu, fried tofu, and tofu skin, were also tested to evaluate the performance of this system. A total of 30 tofu-based samples from different manufacturers were tested by the Grind-N-Go/RRS iiPCR/POCKIT™ Micro system, and the results were compared with side-by-side tested results of the reference RRS rtPCR method with 100 ng DNA prepared by the taco™ mini (silica-based) method (Table 2). With the silica-based DNA extraction then RRS rtPCR method, 13 and 17 tofu samples were positive and negative for RRS, respectively (Table 2). All rtPCR positive samples were also positive by the Grind-N-Go/RRS iiPCR/

Table 4. Retested analyses of discrepant samples for 5 replicates by RRS rtPCR method and RRS iiPCR Reagent Set

No.	rtPCR (Ct)	iiPCR
S2	35.97	+
	36.27	+
	35.70	+
	36.72	+
	36.08	+
S4	ND[1)]	+
	37.98	-
	ND	-
	37.93	-
	ND	+
S13	38.32	-
	ND	+
	39.13	-
	ND	-
	39.24	+
S19	ND	-
	ND	+
	ND	+
	ND	-
	38.74	+
S23	38.43	-
	38.23	-
	37.02	-
	39.44	-
	37.02	+
S24	37.49	+
	36.91	+
	36.76	-
	37.25	+
	36.80	+
T15	ND	-
	39.16	-
	39.15	-
	ND	-
	36.52	+
T17	37.52	-
	39.22	-
	ND	+
	ND	+
	38.84	+
T18	39.66	+
	38.97	+
	ND	-
	38.11	-
	38.62	+

[1)]ND, not determined

POCKIT™ Micro system, but 3 of the 17 rtPCR negative samples (T15, T17, and T18) were positive by the Grind-N-Go/RRS iiPCR/POCKIT™ Micro system. Analysis of the 2x2 contingency table (Table 3) determined that the agreement between two methods was 90% (95% confidence interval: 77.39-100%; kappa value=0.8). The three discrepant samples were further tested in 5 replicates, and all had at least three rtPCR results with Ct values between 36 and 40 and at least one positive result in the Grind-N-Go/RRS iiPCR/POCKIT™ Micro system (Table 4). Similar to observations made with the analyses of the soybean milk samples with the RRS iiPCR Reagent Set, the performance of the Grind-N-Go/RRS iiPCR/POCKIT™ Micro system was comparable to and maybe more sensitive than that of the silica-based DNA extraction then RRS rtPCR method.

The high performance of the Grind-N-Go/RRS iiPCR/POCKIT™ Micro system with tofu-based samples suggested that this system could be applied to RRS monitoring in solid soybean-based products to compliment the rtPCR assay. The combination of a simple and compact nucleic acid extraction device with the user-friendly iiPCR technology allows the system to be performed by users not skilled in molecular biology techniques, making timely informed decisions possible.

In conclusion, the easy-to-use RRS iiPCR Reagent Set alone was demonstrated to be comparable to the reference RRS rtPCR method, which is a time-consuming procedure and requires sophisticated equipment and trained technicians to perform for detecting RRS in soybean-based food samples. For RRS screening purpose, the RRS iiPCR Reagent Set could be used directly for soybean milk. Furthermore, the Grind-N-Go/iiPCR/POCKIT™ Micro system could be completed straightforwardly at point of need to serve as a tool for on-site RRS screening of solid processed foods within 60 min. Furthermore, in combination with various iiPCR reagent sets for other screening-specific markers (such as the CaMV *35S* promoter and the *nos* terminator), the Grind-N-Go/iiPCR/POCKIT™ Micro system should greatly facilitate timely GMO screening for food manufacturers, retailers, and consumers.

Acknowledgments The authors thank GeneReach Biotechnology Corp. for the great assistance for this work.

Disclosure The authors declare no conflict of interest.

References

1. Bawa AS, Anilakumar KR. Genetically modified foods: Safety, risks and public concerns-a review. J. Food Sci. Tech. Mys. 50: 1035-1046 (2013)
2. James C. Global status of commercialized biotech/GM crops: 2014. ISAAA Brief No. 49. ISAAA:Ithaca, NY, USA (2014)
3. Aldemita RR, Reano IM, Solis RO, Hautea RA. Trends in global approvals of biotech crops (1992-2014). GM Crops Food 6: 150-166 (2015)
4. Nikolic Z, Vasiljevic I, Zdjelar G, Ethordevic V, Ignjatov M, Jovicic D, Milosevic D. Detection of genetically modified soybean in crude soybean oil. Food Chem. 145: 1072-1075 (2014)
5. Consumers Union of United States. FAQs about GMOs. It can be tough to figure out why there's so much fuss over genetically modified ingredients in food. Consum. Rep. 80: 12-14 (2015)
6. Samsel A, Seneff S. Glyphosate's suppression of cytochrome p450 enzymes and amino acid biosynthesis by the gut microbiome: Pathways to modern diseases. Entropy 15: 1416-1463 (2013)
7. Van Eenennaam AL. GMOs in animal agriculture: Time to consider both costs and benefits in regulatory evaluations. J. Anim. Sci. Biotechnol. 4: 37 (2013)
8. Pi L, Li X, Cao Y, Wang C, Pan L, Yang L. Development and application of a multi-targeting reference plasmid as calibrator for analysis of five genetically modified soybean events. Anal. Bioanal. Chem. 407: 2877-2886 (2015)
9. Holst-Jensen A. Testing for genetically modified organisms (GMOs): Past, present and future perspectives. Biotechnol. Adv. 27: 1071-1082 (2009)
10. Song Q, Wei G, Zhou G. Analysis of genetically modified organisms by pyrosequencing on a portable photodiode-based bioluminescence sequencer. Food Chem. 154: 78-83 (2014)
11. Chaouachi M, Nabi N, Hafsa AB, Zellama MS, Skhiri F, Saïd K. Monitoring of genetically modified food and feed in the tunisian market using qualitative and quantitative real-time pcr. Food Sci. Biotechnol. 22: 1161-1170 (2013)
12. Miraglia M, Berdal KG, Brera C, Corbisier P, Holst-Jensen A, Kok EJ, Marvin HJ, Schimmel H, Rentsch J, van Rie JP, Zagon J. Detection and traceability of genetically modified organisms in the food production chain. Food Chem. Toxicol. 42: 1157-1180 (2004)
13. Deisingh AK, Badrie N. Detection approaches for genetically modified organisms in foods. Food Res. Int. 38: 639-649 (2005)
14. Elsanhoty R. Genetically modified roundup ready soybean in processed meat products in the kingdom of saudi arabia. Ann. Agric. Sci. 58: 231-237 (2013)
15. Taiwan Food and Drug Administration (TFDA). Method of test for genetically modified foods – event-specific qualitatively and quantitatively test of soybean event 40-3-2 (RRS) (2013)
16. Bonfini L, Van den Bulcke MH, Mazzara M, Ben E, Patak A. Gmomethods: The european union database of reference methods for gmo analysis. J. AOAC Int. 95: 1713-1719 (2012)
17. Holst-Jensen A, Ronning SB, Lovseth A, Berdal KG. Pcr technology for screening and quantification of genetically modified organisms (gmos). Anal. Bioanal. Chem. 375: 985-993 (2003)
18. Wang C, Li R, Quan S, Shen P, Zhang D, Shi J, Yang L. Gmo detection in food and feed through screening by visual loop-mediated isothermal amplification assays. Anal. Bioanal. Chem. 407: 4829-4834 (2015)
19. Feng J, Tang S, Liu L, Kuang X, Wang X, Hu S, You S. Development of a loop-mediated isothermal amplification (lamp) assay for rapid and specific detection of common genetically modified organisms (gmos). Int. J. Food Sci. Nutr. 66: 186-196 (2015)
20. Tsai YL, Wang HC, Lo CF, Tang-Nelson K, Lightner D, Ou BR, Hour AL, Tsai CF, Yen CC, Chang HF, Teng PH, Lee PY. Validation of a commercial insulated isothermal pcr-based pockit test for rapid and easy detection of white spot syndrome virus infection in litopenaeus vannamei. PLoS One 9: e90545 (2014)
21. Krishnan M, Ugaz VM, Burns MA. Pcr in a rayleigh-benard convection cell. Science 298: 793 (2002)
22. Tsai YL, Lin YC, Chou PH, Teng PH, Lee PY. Detection of white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions. J. Virol. Methods 181: 134-137 (2012)
23. Tsai YL, Wang HT, Chang HF, Tsai CF, Lin CK, Teng PH, Su C, Jeng CC, Lee PY. Development of taqman probe-based insulated isothermal pcr (iipcr) for sensitive and specific on-site pathogen detection. PLoS One 7: e45278 (2012)
24. Ambagala A, Pahari S, Fisher M, Lee PA, Pasick J, Ostlund EN, Johnson DJ, Lung O. A rapid field-deployable reverse transcription-insulated isothermal polymerase chain reaction assay for sensitive and specific detection of bluetongue virus. Transbound. Emerg. Dis. [Epub ahead of print] (2015)
25. Tsen HY, Shih CM, Teng PH, Chen HY, Lin CW, Chiou CS, Wang HT, Chang HF, Chung TY, Lee PY, Chiang YC. Detection of *Salmonella* in chicken meat by insulated isothermal PCR. J. Food Protect. 76: 1322-1329 (2013)
26. Balasuriya UB, Lee PY, Tiwari A, Skillman A, Nam B, Chambers TM, Tsai YL, Ma LJ, Yang PC, Chang HF, Wang HT. Rapid detection of equine influenza virus H3N8 subtype by insulated isothermal RT-PCR (iiRT-PCR) assay using the POCKIT™ nucleic acid analyzer. J. Virol. Methods 207: 66-72 (2014)
27. Wilkes RP, Tsai YL, Lee PY, Lee FC, Chang HF, Wang HT. Rapid and sensitive detection of canine distemper virus by one-tube reverse transcription-insulated isothermal polymerase chain reaction. BMC Vet. Res. 10: 213 (2014)
28. Wilkes RP, Lee PY, Tsai YL, Tsai CF, Chang HH, Chang HF, Wang HT. An insulated isothermal pcr method on a field-deployable device for rapid and sensitive detection of canine parvovirus type 2 at points of need. J. Virol. Methods 220: 35-38 (2015)
29. Wilkes RP, Kania SA, Tsai YL, Lee PY, Chang HH, Ma LJ, Chang HF, Wang HT. Rapid and sensitive detection of feline immunodeficiency virus using an insulated isothermal PCR-based assay with a point-of-need PCRdetection platform. J. Vet. Diagn. Invest. 27: 510-515 (2015)
30. Lung O, Pasick J, Fisher M, Buchanan C, Erickson A, Ambagala A. Insulated isothermal reverse transcriptase pcr (iiRT-PCR) for rapid and sensitive detection of classical swine fever virus. Transbound. Emerg. Dis. [Epub ahead of print] (2015)
31. European Network of GMO Laboratories (ENGL). Definition of minimum

performance requirements for analytical methods of GMOtesting (2015)
32. Prins TW, van Dijk JP, Beenen HG, Van Hoef AA, Voorhuijzen MM, Schoen CD, Aarts HJ, Kok EJ. Optimised padlock probe ligation and microarray detection of multiple (non-authorised) GMOs in a single reaction. BMC Genomics 9: 584 (2008)
33. James D, Schmidt AM, Wall E, Green M, Masri S. Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. J. Agr. Food Chem. 51: 5829-5834 (2003)
34. Datukishvili N, Kutateladze T, Gabriadze I, Bitskinashvili K, Vishnepolsky B. New multiplex PCR methods for rapid screening of genetically modified organisms in foods. Front. Microbiol. 6: 757 (2015)