

## The used of recombinant plasmid DNA in GMO quantitative analysis of insect resistance maize targeted unapproved StarLink corn and approved Bt176 corn in food and feed sold commercially sold Malaysia

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**Abstract:** Genetically modified organisms (GMO) are increased remarkably from year to year and the estimated global area cultivated with genetically modified (GM) crops reached 125 million hectares in year 2008. However, insect resistance maize based on *Bacillus thuringiensis* (Bt) is of the most cultivated GM crop in worldwide. *Bacillus thuringiensis* (Bt) is an aerobic, gram-positive bacterium that synthesize one or more Cry protein that are toxic to various types crop and forestry insects pests. To date, several cry genes have been introduced into GM plant to combat with various type of insect. Worldwide commercialization of GM crops has raised the customers' concern about the Biosafety issues, and thus, many countries have implemented the labeling legislations for GM food and their derivatives. In this study, we introduced the quantitative analysis method based on the recombinant plasmid DNA as calibrators that can be used to determine the percentage of GMO content in various types of food and feed samples. Therefore, we have reported 7.5% (6/80) of the samples were contained StarLink maize and 1.25% (1/80) samples were contained Bt176 maize. Additionally, the percentage of GM content in each positive sample were further determined with the developed quantitative method. The percentage of the StarLink corns that present in the positive samples were varies from 0.09% to 2.53% and Bt176 corn that present in the positive sample was 16.90%. The present study demonstrated that the recombinant plasmid DNA that used in quantitative real-time method as good alternative quantitative analysis of GM content.

**Keywords:** genetically modified organisms, StarLink corn, Bt176 corn, SYBR Green I, quantitative real-time PCR, recombinant plasmid DNA

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### Introduction

Genetically modified organism (GMO) is defined as organism whose genome has been altered unnaturally by the genetic engineering techniques. Recently, the rapid development in recombinant DNA technology enabled the introduction of exogenous genes that confer to a particular trait into plant genome. This development of GM crops hold pace to four general purposes, to increase yield and productivity in which beneficial to agricultural; to reduced reliance on herbicides and pesticides by making GM crops that tolerance to herbicide and resistance to insect; to enhance the nutritional quality of crop; disease prevention in which food may serve as vaccines (Karamollaoğlu et al., 2009). In 2008, the global area cultivated with GM crops increased significantly reaching 125 million hectares, and thus, the use of GMO as food, food ingredient and

feed is become more and more common (James, 2008). Additionally, insect-resistance maize based on *Bacillus thuringiensis* (Bt) is one of the widely used trait in planting GM crops (Dinon et al., 2008). However, GM crops for human consumption and animal feed has raised scientific and public debate concerning their safety and potential adverse effect. The important risks concerning over GMO are food allergenicity, potential gene transfer to non-target organisms, induction of antibiotic resistance and destruction of biodiversity (Michellini et al., 2008).

Recently, numerous cases have been reported about the occurrences of unapproved GM lines that released into the market sporadically (Mano et al., 2009). Particular attention should pay to Maize line CBH-351 (trade name StarLink) corn case. StarLink corn is insect-resistance corn encodes for modified *cry9C* gene from *Bacillus thuringiensis* subsp. *tolworthi* that confer resistance to feeding damage of

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lepidoteran insects, for example, the European corn borer (Windels et al., 2003). Due to the Cry9C protein is not fully digested in vitro and may cause potential allergenicity to human, it was approved by USA FDA in 1998 only for use as animal feed and not for human consumption (Yonemochi et al., 2003). A crucial aspect in the traces of *cry9C* was not only detected in the food supply in US and present in around 10% of US corn crop, but, it had been detected in imported corn and corn product in Japan, Korea, Nicaragua, and Mexico (Jennifer, 2008).

Under these circumstances, the legislation is regulated strictly by the government of some countries become primary important both to impede the entry of unapproved GM lines into the market and to protect their consumers' right to the information about the product they consumed daily. Different countries have implemented their own legislation in which elaborated authorization of GM product, cultivation of GM crop, traceability and labeling as well as post-market surveillance for the occurrence of transgene in food, food ingredient and animal feed. The stakeholders of the food and feed supply are necessary to comply with legislation in order to commercialize and export the food and feed product globally (Hamels et al., 2009). Nevertheless, the European Union (EU) has implemented more stringent policies, for example, Regulation (EC) No. 1829/2003 for labeling and Regulation (EC) No. 1830/2003 for the traceability of the GMO in food (Hamels et al., 2009). For example, mandatory label is needed if the authorized GM product content exceeds a 0.9% in any of food and feed products. However, the threshold is set to a maximum of 0.5 % for non-authorized GM ingredients in a food or feed provided particular GMO possess a positive safety assessment for health and the environment (Karamollaoğlu et al., 2009). Additionally, the threshold is set to 0% for seed, which mean all GM seed must be labeled based on 2001/18/EC (Marmirolo et al., 2008). Therewithal, the threshold level for GM labeling varies in different countries, for example, 0.9% in Russia, 1% in Australia, New Zealand, and Saudi Arabia, 3 % in South Korea as well as 5% in Indonesia, Japan, and Taiwan (Marmirolo et al., 2008). In Malaysia, the Biosafety Bill 2006 was passed by the Parliament in July 2007 and mandatory labeling is reckoned to be implemented soon.

Various detection systems either based on DNA or protein has been developed to facilitate the enforcement of legislation. Additionally, the regulation of threshold values has necessitated the development of reliable and accurate quantitative systems that allow more numerical information. Also,

validated detection methods can be used by biotech companies to commercialize their products on Market (Milcamps et al., 2009). Majority of the developed detection method is DNA-based techniques as DNA is relatively stable analyte for both raw materials and highly processed food. However, DNA-based method is a good alternative to protein-based method due to highly stability of DNA can be extracted from all most any kind of biological samples. Contrasting to DNA, protein is often degraded during the various industrial food and feed processes. Generally, polymerase chain reaction (PCR) is the most accepted screening and detection methods due to its high sensitivity and specificity. However, end-point PCR has drawback of lack of quantitative information leading to the establishment of real-time PCR that monitors amplification of target sequence in every cycle by using fluorescent dye. The fluorescent dye, such as SYBR Green, is coupled to PCR products that formed at each cycle during amplification. The fluorescence signal that collected is proportional to amount of the PCR products generated during exponential phase. The relative amount of transgene can be determined based on the ratio between target-specific sequence and a plant-specific reference gene. Lately, real-time polymerase chain reaction is one of the methods that used to test labeling compliance of food and feed with EU regulation 1829/2003 (Jasbeer et al., 2009; Gaudron et al., 2009).

In this study, we firstly explore the qualitative analysis of GMO content in the collected samples and the Recombinant plasmid DNA as calibrator in quantitative analysis. Both insect resistance StarLink corn and Bt176 corn will be identify in the various food sample. The purpose of this study was to develop and establish a simple and reliable quantitative method to determine the percentage target GMO that present in the maize containing foods, such as raw maize and processed food, as well as, animal feed in Malaysian market.

## Materials and Methods

### *Samples*

For the construction of the recombinant plasmid DNA as calibrators, certified reference materials (CRM) consisting of dried maize powder of 2% Bt176 maize and StarLink Corn, developed by the Institute for Reference Materials and Measurements (IRMM, Belgium) and commercialized by Fluka. In addition, this CRM were also utilized as positive control in both qualitative and quantitative analysis. Various types of maize-derived food products and animal feeds were randomly collected from the local

supermarkets, traditional markets and grocery stores in Malaysia.

#### *DNA extraction with cetyltrimethylammonium bromide (CTAB) method*

Genomic DNA was isolated from all the collected samples using the protocol illustrated in previous study (Mafra et al., 2008), with some modification. Firstly, 100 mg/samples were ground into fine powder using a mortar and a pestle, whereas semi-solid samples were ground under liquid nitrogen. Then, 0.5 mL of CTAB extraction buffer (20 g CTAB/L, 1.4 M NaCl, 0.1 M Tris-HCl and 20 mM EDTA) was added into the fine powder samples and mixed thoroughly by force of vortex and eventually mixture was incubated for 1 hr at 65°C, with occasional stirring. Next, after centrifugation (10 min, 16,000 g) of suspension, supernatant was collected. 200 µL of chloroform was added into the supernatant and mixed thoroughly by vortex. Mixture was centrifuged (10 min, 14,000 g) and the upper layer was transferred into a new tube containing double volume of CTAB precipitation solution (5 g/L, 0.04 M NaCl), the mixture was incubated for 1 hr at room temperature. Then, the mixture was centrifuged (10 min, 14,000 g), the supernatant was removed and the precipitate was dissolved in 350 µL 1.2 M NaCl and 350 µL chloroform, mixed by vortex. After centrifugation (10 min, 14,000 g), the upper layer was transferred to a new tube containing same volume of isopropanol, then incubated overnight at -20°C. The following day, mixture was centrifuged (10 min, 14,000 g) at 4°C, the collect pellet was washed with 50 µL of ethanol solution (70% v/v). Finally, the pellet was dried and eluted in 100 µL of sterile ultrapure water. DNA purity and concentration were determined by Biophotometer.

#### *Qualitative analysis with polymerase chain reaction*

A qualitative PCR method for detection of maize-specific DNA was performed by using the species specific invertase primers IVR1 (5'-CCGCTGTATCACAAGGGCTGGTACC-3') and IVR2 (5'-GGAGCCCGTGTAGAGCATGACGATC-3'), yielding amplicon length of 226 bp. Primers IVR1/IVR2 are suggested by International Organization for Standardization (ISO) (Cazzola and Petruccioli, 2006). The CDPK promoter/*cry1Ab* gene specific identification to Bt 176 corn was analyzed by using primer pairs CRY03 (5'-CTCTCGCCGTTTCATGTCGGT-3') and CRY04 (5'-GGTCAGGCTCAGGCTGATGT-3'), yielding amplicon length of 211 bp (Lutz

et al., 2006). The primer pairs CBH1-5'(5'-TATTACTTCAGCCATAACAAAAGAACTCT-3') and CBH1-3'(5'-GTAGCTGTCGGTGTAGTCCTCGT-3') were specific to identify the StarLink Corn, yielding amplicon length of 103 bp (Matsuoka et al., 2001). Only sample that positive to primer pairs IVR1/IVR2 were subjected to the identification of Bt176 corn and StarLink Corn by using primer pairs CRY03/CRY04 and CBH1-5'/CBH1-3', respectively. Qualitative PCR was carried out in a Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) in a final volume of 20 µL, containing of 2 µL of DNA extract, 1× PCR buffer (10 mM Tris-HCl (pH8.3), 50 mM KCl and 2 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 1.5 units of i-Taq™ DNA polymerase (Intron, Gyeonggi-do, Korea), 0.125 µM of primers. PCR condition was according to amplification cycles as follow: predenaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 45 sec, annealing beginning at 65°C and ending at 55°C for 30 sec, and extension at 72°C for 45 sec, and extension at 72°C for 7 mins. After PCR amplification, 20 µL of amplified PCR product was analyzed by 1.8 % agarose gel electrophoresis and stained with ethidium bromide (0.5 µg/ml). The agarose gel was visualized using a UV transilluminator (Alpha Imager, Alpha Innotech, USA).

#### *Construction of recombinant plasmid DNA as calibrators*

The DNA calibrators that used for quantitative analysis of this study were based on the constructed Recombinant plasmid DNA. Three sets of Recombinant plasmid DNA were constructed which are the endogenous maize species specific Invertase sequence, the CDPK promoter/*cry1Ab* sequence specific to Bt176 corn and *cry9C* sequence specific to StarLink Corn. PCR reactions were performed on maize DNA isolate from Bt176 corn and StarLink corn from CRMs, IRMMs, in order to yield sufficient amount of PCR product for cloning of invertase gene, *cry1Ab* gene and *cry9C* gene. The amplified PCR products were separated by preparative agarose gel; the specific fragments were then eluted from the gel with Gene<sup>∇</sup>All™ Gel Extraction SV kit (Seoul, Korea). The PCR products were confirmed with direct sequencing analysis. Subsequently, the PCR products were each cloned into the pDrive cloning vector with Qiagen® PCR Cloning Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. Individual clones, selected from the selective Luria Bertani (LB) medium, were then subjected to the plasmid DNA isolation with Fast Plasmid Mini Kit (Eppendorf AG, Hamburg, Germany) and analyzed

by Restriction enzyme *Eco*R1 digest.

#### *Quantitative analysis with SYBR green based real-time PCR*

The real-time PCR reactions were carried out in a special glass capillaries specially design for used in LightCycler® 1.2 carousel based instrument (Roche Diagnostic, Germany). SYBR-based quantitative analysis was performed with Quantitect SYBR Green Kit (Qiagen, Hilden, Germany) in final volume of 20 µL consisted of 2 × QuantiTect SYBR Green mixtures, 0.5 µM of each primer, 1 µL of DNA and 7 µL of RNase-free water. The real-time PCR condition was performed with predenaturation for 15 min at 95°C, followed by 40 cycles of amplification and quantification with 15 s at 94°C, 10 s at 59°C and 10 s at 72°C. Subsequently, the melting curve analysis was carried immediately after the PCR amplification which the temperature was gradually raised from 72°C to 90°C at heating 0.2°C/s with a continuous fluorescence measurement and eventually, cooling step to 40°C. Finally, the data was analyzed and generated with LightCycler® 1.2 software version 4.05 (Roche Diagnostic, Germany).

#### *Construction of calibration curves*

Three calibration curves was generated from the constructed recombinant plasmid DNA by carried out serial dilutions to obtain the concentration range of 0.01 ng/ µl to 100 ng/ µl. All the real-time PCR were carried out in triplicate for each of concentration. Then, the calibration curves were generated based on the fluorescent signal emitted by plotting the Cp values against the logarithm of the concentration in ng/ µl. Quantitative analysis was carried out with 'Fit-Point Method'. Finally, the constructed calibration curves were validated with the genomic DNA from Bt176 corn and StarLink Corn from CRM, IRMM.

#### *Determination of cry1Ab and cry9C content in collected samples*

The *cry1Ab* gene sequence and maize invertase gene sequence were amplified in two separate capillaries during the same run. However, *cry9C* gene sequence and maize invertase gene sequence were amplified in two separate capillaries during the same run. According the constructed calibration curve, Cp value for each sample was used to determine the amount of GM content and endogenous gene. Therefore, the percentage of GM content was calculated by dividing the amount of target specific transgene by that of the plant species specific endogenous gene and then multiplying by 100%. Each of the samples was repeated twice in order to estimate the precision of quantitative results from

standard deviations and relative standard deviation. However, positive control and negative control was included in every run to ensure the reliability of this assay. Positive control consisted of genomic DNA from CRM, IRMM and negative control was sterile ultrapure water.

## **Results and Discussions**

#### *Qualitative analysis with polymerase chain reaction*

A total of 101 samples that containing maize were collected randomly from the Malaysia market. Various types of samples were collected which consist of raw maize (6), processed food (46) and vegetarian food (3). Besides, different types of animal feeds were consisted of common animal feed (39), rabbit feed (4), chicken (3) and pig (1). All the collected samples were subjected to DNA isolation by using the CTAB method and subsequently analyzed qualitative by PCR method. In this study, we demonstrated 80 out of 101 samples were successfully extracted and positive to maize invertase gene (226 bp). However, we also demonstrated 6 samples were found positive to target specific *cry9C* gene (103 bp) and 1 sample was positive to target specific *cry1Ab* gene (211 bp). Figure 1 shown results of qualitative PCR analysis of maize invertase gene, *cry9C* gene and *cry1Ab* gene in various types of samples. The amplified PCR product of maize invertase gene sequences (226 bp), *cry9C* gene sequences (103 bp) and *cry1Ab* gene sequences (211 bp) was separated and visualized in agarose gel electrophoresis. Figure 2 shown the representative agarose gel electrophoresis for maize invertase gene sequences at 226 bp.

#### *Setup of calibration curves*

Three sets of cloned plasmid DNA was successfully constructed in this study. The amplified PCR products for *cry9C* and *cry1Ab* gene sequences that extracted from the agarose gel were confirmed by sequencing Blast result for gel extract for primer pair CBH1-5'/CBH1-3' indicated that 96% homology with *Zea mays* transgenic clone pRVA9909 *cry9C* gene. However, blast result for gel extract for primer pairs CRY03/CRY04 indicated 95% homology with synthetic construct *cry1AC* gene from *Bacillus thuringiensis* and 100% homology with *Zea mays* calcium-dependent protein kinase (CDPK) gene. Additionally, the recombinant plasmid DNA for maize invertase gene sequences from primer pairs IVR1/IVR2 was confirmed by sequencing. Blast result has shown 93% homology with *Zea mays* soluble acid invertase IVR1 mRNA. Next, all three set of recombinant plasmid DNA were further analyzed by Restriction enzyme *Eco*R1 digest and the mixture

was analyze by 1.5% agarose gel electrophoresis. The expected amplicon length of 226 bp, 211 bp and 103 bp for maize invertase gene sequence, *cry1Ab* gene sequence and *cry9C* gene sequence, respectively, were observed.

In quantitative analysis, three calibration curves were constructed for maize invertase gene as plant species reference, *cry1Ab* gene targeted Bt176 corn and *cry9C* gene targeted StarLink corn. A set of serial dilution between 0.01 ng/ µl and 100 ng/ µl. The PCR efficiencies were calculated by the LightCycler® software version 4.05 based on the slope of the calibration curves. The PCR efficiencies were range from 1.84 to 2.097 which closed to 2. The errors of the calibration curve were between 0.00358 and 0.0566. Hence, both good PCR efficiency and low error value that obtained suggested the constructed calibration curves were very suitable for quantitative analysis of GM content. In addition, the genomic DNA extracted Bt176 corn and StarLink corn from CRM, IRMM were used as to validate the accuracy of the established quantitative system by calculating the percentage of the GM content and compared to true value. In this study, we demonstrated the percentage of GM content in all three repeated StarLink corn CRM standard were 5.962%, 5.538% and 4.421% with the errors of 19.24%, 10.76% and 11.58% as shown in table 1. Thus, this system was suitable to be used in StarLink Corn content quantification. However, we also demonstrated the percentage of GM content in two repeated Bt176 corn CRM standard were 2.25% and 1.817% with calculated errors of 12.5% and 9.15% as shown in table 2. Consequently, this established system was suitable to be used as quantitative analysis of Bt176 content.

*Quantitative analysis with SYBR green based real-time PCR using recombinant plasmid DNA as a calibrator*

The measurement of GMO material was based on percentage genome/genome or percentage weight/weight (Tavernier et al., 2001). In this study, we determined the percentage of GM content in the unknown samples. The relative quantification

analysis of the GM content was referred to the two independent absolute quantifications of the plant specific references gene and target specific transgene. There are 6 samples and 1 sample were found positive to StarLink corn and Bt176 corn were further subjected to quantitative real-time PCR assay in order to identify the % of GM content in the samples. For quantitative analysis of StarLink corn for maize invertase gene shown average Cp values varies from 24.25 to 29.06 with standard deviation of 0.12 to 0.99, whereas *cry9C* gene sequence gave average Cp values of 29.91 to 38.02 with standard deviation of 0.06 to 2.57. Referring to the quantitative results shown in table 3, the percentage of StarLink corn content for all the 6 samples were varies from 0.09% to 2.53% with standard deviation varies from 0.0050 to 2.6789. However, the quantitative analysis of Bt176 corn for maize invertase gene gave average Cp values of 23.95 and 30.92 with standard deviation of 0.14 to 0.63, whereas *cry1Ab* gene sequence gave average Cp values of 28.92 and 32.69 with standard deviation of 0.16 and 0.27. According to table 4, the percentage of Bt176 corn content in animal feed was 16.90% with standard deviation of 8.4125.

Melting curves that performed immediately after the Real-time PCR amplification was particularly important to demonstrate the primer specificity and to ensure the reliability of the developed quantitative system. The melting curves described one peak for primer pair IVR1/IVR2, primer pair CBH1-5'/CBH1-3' and primer pair CRY03/CRY04 was presented in Figure 3, figure 4 and figure 5, respectively.

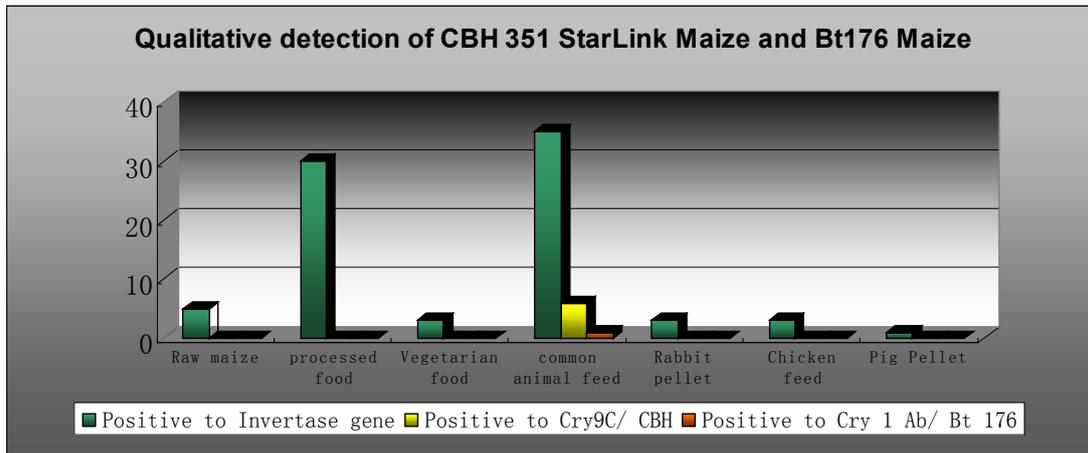
The CTAB extraction method that used in this study exhibited a very suitable method to extract amplified DNA especially in a huge number of samples. Although this method was very cost effective and can be used in various types of samples, it was extremely time-consuming. This statement was previously suggested that the CTAB method presents the disadvantage of being time consuming; however, it produces less degraded DNA from the various types of samples (Mafra et al., 2008). In the qualitative PCR analysis, primer pair IVR1/IVR2 was used, first

**Table 1.** Statistical analysis of accuracy for the quantitative system for StarLink corn.

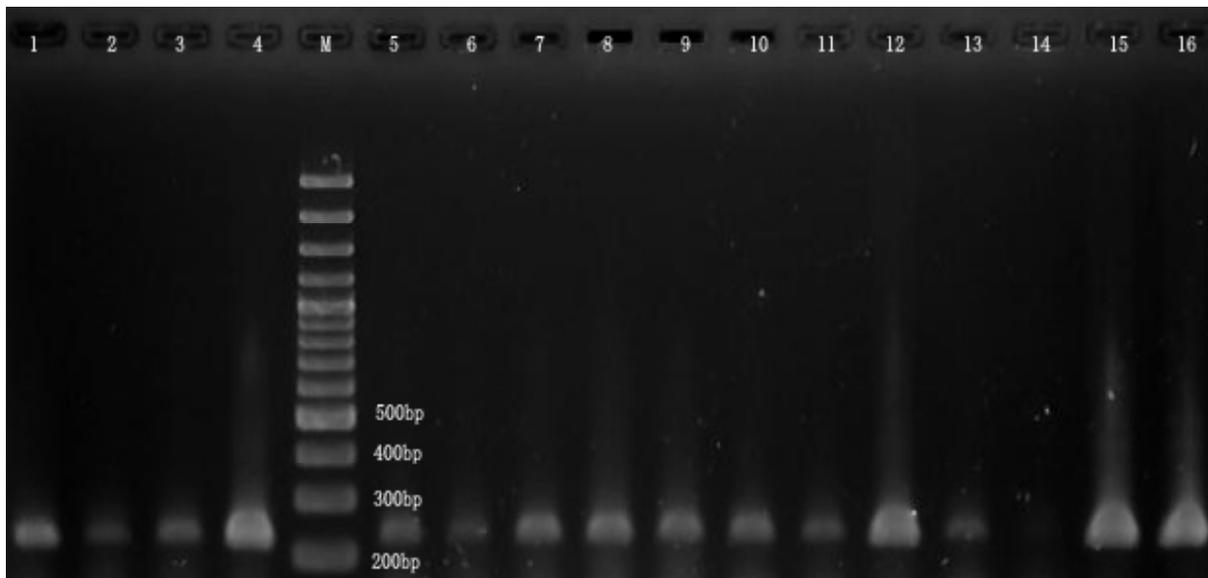
5% StarLink corn from CRM	True value (%)	Calculated StarLink (%)	% of error/bias
Set 1	5	5.962	19.24
Set 2	5	5.538	10.76
Set 3	5	4.421	11.58

**Table 2.** Statistical analysis of accuracy for the quantitative system for Bt176 corn.

2% Bt176 corn from CRM	True value (%)	Calculated Bt176 (%)	% of error/bias
Set 1	2	2.25	12.5
Set 2	2	1.817	9.15



**Figure 1.** Qualitative PCR analysis of maize invertase gene, *cry9C* gene and *cry1Ab* gene in various types of samples. 6 samples from common animal feeds were found positive to *cry9C* gene and 1 sample from pig feed was positive to *cry1Ab* gene.



**Figure 2.** Representative agarose gel electrophoresis of PCR analysis of DNA extracted from animal feed samples with maize invertase gene (226bp). Lane M, 100bp DNA ladder; Lane 1-16, animal feeds.

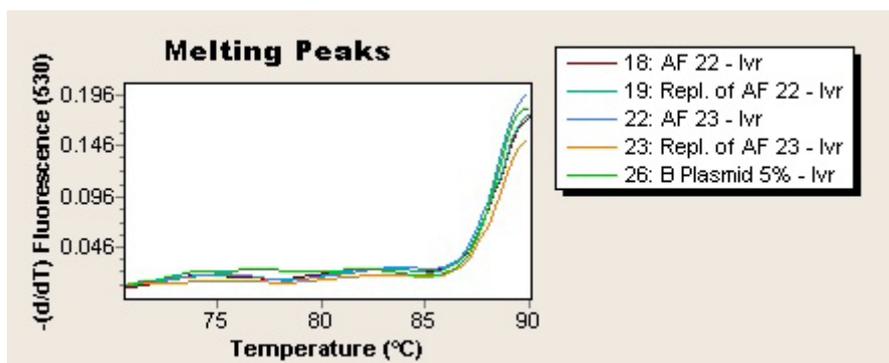


Figure 3. Melting Peaks curves from the SYBR Green assay in LightCycler® real-time PCR system using primer pair IVR1/IVR2.

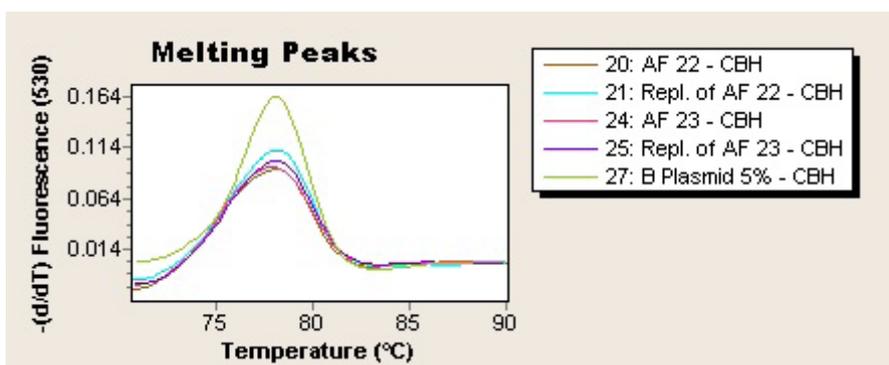


Figure 4. Melting Peaks curves from the SYBR Green assay in LightCycler® real-time PCR system using primer pair CBH 1-5'/CBH1-3'.

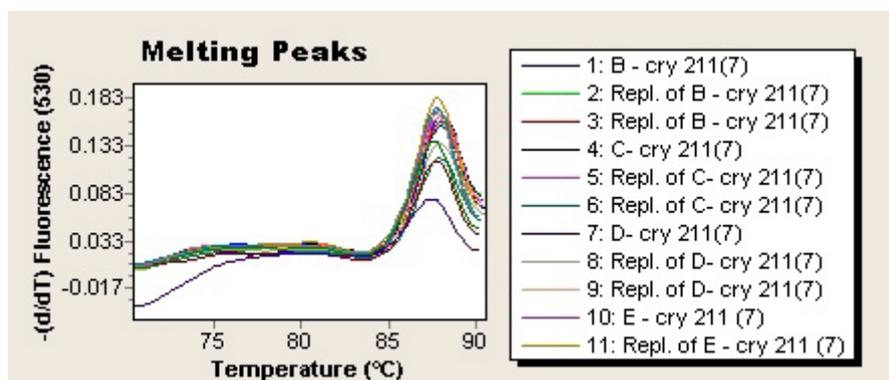


Figure 5. Melting Peaks curves from the SYBR Green assay in LightCycler® real-time PCR system using primer pair CRY03/CRY04.

**Table 3.** Determination of the StarLink content in 6 maize samples that detected positive in qualitative PCR analysis with primer CBH1-5'/CBH1-3', by LightCycler Real-Time PCR using the QuantiTect SYBR Green PCR Kit.

No	Sample	Invertase fragment sequence		Cry 9C gene sequence		Calculated		Mean	STD %
		Cp value	Conc	Cp value	Conc	StarLink corn content %	StarLink corn content %		
1	AF1	27.80	0.000718	37.46	0.000003570	0.497	0.61	0.1560	
2	AF1	26.97	0.00123	35.98	0.000008830	0.718	0.57	0.1212	
3	AF2	28.24	0.00054	37.96	0.000002630	0.487	2.53	2.6789	
4	AF2	27.95	0.000653	37.15	0.000004300	0.658	0.10	0.0209	
5	AF3	29.27	0.000274	38.64	0.000001730	0.631	4.98	0.7903	
6	AF3	28.85	0.000362	35.01	0.000016000	4.420	0.37	0.0050	
10	StarLink CRM standard	9.01	158	13.30	9.420000000	5.962	0.09	0.0075	
11	AF17	26.53	0.00165	38.54	0.000001874	0.114	0.085		
12	AF17	25.13	0.00413	37.50	0.000003470	0.084			
15	StarLink CRM standard	24.62	0.00576	30.12	0.000319000	5.538			
16	StarLink CRM standard	23.88	0.00932	29.70	0.000412000	4.421			
17	AF22	25.01	0.00374	34.05	0.000013800	0.369			
18	AF22	24.84	0.0042	33.89	0.000015200	0.362			
19	AF23	23.87	0.00793	35.03	0.000007580	0.096			
20	AF23	23.76	0.00851	35.11	0.000007230	0.085			

**Table 4.** Determination of the Bt176 corn content in 1 maize sample that detected positive in qualitative PCR analysis with primer CRY03/CRY04, by LightCycler Real-Time PCR using the QuantiTect SYBR Green PCR Kit.

Sample	Invertase fragment sequence		Cry1Ab gene sequence		Calculated	Mean		STD %
	Cp value	Conc	Cp value	Conc		Bt176 corn content %	Bt176 corn content %	
AF28	31.36	0.766	32.59	0.175000000	22.846	16.90	8.4125	
AF28	30.47	1.37	32.80	0.150000000	10.949			
Bt176	24.05	91.7	28.73	2.460000000	2.683	2.25	0.6119	
Bt176	23.86	104	29.10	1.890000000	1.817			

to exhibit the amplifiable maize DNA in the isolated sample and second to identify the isolated sample contained maize specific invertase gene sequences. This study concluded that 78% of genomic DNA was successfully extracted from the various types maize samples. 80 samples that positive to maize invertase gene sequences were subjected to qualitative analysis of target specific transgene which are *cry9C* gene sequences and *cry1Ab* gene sequences. Fortunately, there are only the 6 animal feeds and 1 animal feed demonstrated the present of StarLink corn and Bt176 corn, respectively. As a result, in this study, we are glad to report that there were absent of both StarLink corn and Bt176 corn in human consume food products in Malaysian market.

Generally, there are two choices of calibrators were used to generate the calibration curves in quantitative analysis, the genomic DNA from CRMs (Terry and Harris, 2001) as well as cloned plasmid DNA fragments (Zhang et al., 2008). As described previously, cloned plasmid DNA fragment represents a very good alternative as compare to CRM DNA standard, in terms of, broad dynamic range as CRM DNA standards has limited quantitative range of 0-5% (Burns et al., 2006), as well as inconvenient preparation procedures and expensive. Again, cloned DNA fragment provides a cost effective, long term stability and flexibility (Tavernier et al., 2001). Both BLAST results and restriction enzyme digest analysis that mentioned above suggested the constructed recombinant plasmid DNA can be used as calibrators in setting the calibration curves in quantitative analysis.

In quantitative real-time PCR, SYBR Green based detection format is most commonly used method because it was more economical and easier in terms of experimental design as compare to the other detection format such as Taqman probe and molecular beacons (Andersen et al., 2006). In addition, the melting curve can be performed immediately after the amplification cycle in order to examine the specificity of the assay. Several researchers worldwide reported that SYBR Green based assay was extremely sensitive for the short amplicon that exhibits cleanest one peak melting curve for each primers set (Andersen et al., 2006). In this study, we have established a suitable calibration curve with high PCR efficiency and low error values reported in all three set of calibration curves. The developed quantitative system for StarLink Corn and Bt176 corn also validate by CRM DNA standard by calculating the percentage of errors. As referred to previous researcher reported the errors of the quantitative results were varies from 0.67 to 28.00% (Yang et al., 2007) and 0.60 to 8.78% (Zhang et al.,

2008). Therefore, the percentage error that reported in this study was relatively low, and thus, this developed system can be used in quantitative analysis.

Cultivation of GM crops increased dramatically in every year led to the used of GM crops for human consumption and animals more and more common. Under these circumstances, labeling of GMO content in food and animal feed exceeds certain threshold level was implemented in many countries. Currently, Real-time PCR is method of choices to be used in compliance with GMO labeling of food and feed with EU regulation 1829/2003 (Gaudron et al., 2009). Quantitative system that developed in this study can be used to provide numerical data of GMO content that express in percentage. The LightCycler® 1.2 carousel-based is one of the fastest real-time PCR instruments that allowed the completion of real-time PCR assay less than two hours. Both insect-resistance maize, unapproved Starlink corn and approved Bt176 corn, were focused in this study. The percentage of StarLink corn that present in the six animal feeds were 0.09 %, 0.10%, 0.37%, 0.57%, 0.61%, and 2.53%. According to the EU regulation (EC) No. 1829/2003, the threshold level for unapproved GM content of 0.5%, there are five samples must be labeled. However, one animal feed that shown positive to Bt 176 maize shown very high Bt176 content with 16.90% also required labeling.

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### References

- Andersen, C. B., Holst-Jensen, A., Berdal, K. G., Thorstensen, T. and Tengs, T. 2006. Equal performance of Taqman, MGB, Molecular Beacon, and SYBR green-based detection assays in detection and quantification of roundup ready soybean. *Journal of Agricultural and Food Chemistry* 54: 9658-9663.
- Burns, M., Corbisier, P., Wiseman, G., Valdivia, H., McDonald, P., Bowler, P., Ohara, K., Schimmel, H., Charels, D., Damant, A. and Harris, N. 2006. Comparison of plasmid and genomic DNA calibrants for the quantification of genetically modified ingredients. *European Food Research and Technology* 224: 249-258.

- Cazzola, M. L. and Petruccioli, S. 2006. Semiquantitative analysis of genetically modified maize and soybean in food. *Electronic Journal of Biotechnology*. 9: 320-325.
- Dinon, A. Z., de Melo, J. E. and Arisi, A. C. M. 2008. Monitoring of MON810 genetically modified maize in foods in Brazil from 2005 to 2007. *Journal of Food Composition and Analysis*. 21: 515-518.
- Gaudron, T., Peters, C., Boland, E., Steinmetz, A. and Moris, G. 2009. Development of a quadruplex-real-time-PCR for screening food for genetically modified organisms. *European Food Research and Technology* 229: 295-305.
- Hamels, S., Glouden, T., Gillard, K., Mazzara, M., Debode, F., Foti, N., Sneyers, M., Nuez, T. E., Pla, M., Berden, G., Moens, W., Bertheau, Y., Audéon, C., Van den Eede, G. and Remacle, J. 2009. A PCR-microarray method for the screening of genetically modified organisms. *European Food Research and Technology* 228: 531-541.
- James, C. Executive summary of global status of commercialized biotech/GM crops: 2008. The First Thirteen Years, 1996 to 2008. ISAAA Briefs 2008, No.39.
- Jasbeer, K., Son, R., Mohamad Ghazali, F. and Cheah, Y. K. 2009. Real-time PCR evaluation of seven DNA extraction methods for the purpose of GMO analysis. *International Food Research Journal* 16: 329-341.
- Jennifer, C. 2008. Illegal GMO releases and corporate responsibility: Questioning the effectiveness of voluntary measures. *Ecological Economics* 66: 348-358.
- Karamollaoglu, İ., Öktem, H. A. and Mutlu, M. 2009. QCM-based DNA biosensor for detection of genetically modified organisms (GMOs). *Biochemical Engineering Journal* 44, 142-150.
- Lutz, B., Wiedemann, S. and Albrecht, C. 2006. Degradation of transgenic *cryIAb* DNA and protein in Bt176 maize during the ensiling process. *Journal of Animal Physiology and Animal Nutrition* 90: 116-123.
- Mafta, I., Silva, S. A., Moreira, E. J. M. O., da Silva, C. S. F., Beatriz, M. and Oliveira, P. P. 2008. Comparative study of DNA extraction methods for soybean derived food products. *Food Control* 19: 1183-1190.
- Mano, J., Shigemitsu, N., Futo, S., Akiyama, H., Teshima, R., Hino, A., Furui, S. and Kitta, K. 2009. Real-time PCR Array as a Universal Platform for the detection of genetically modified crops and its application in identifying unapproved genetically modified crops in Japan. *Journal of Agricultural and Food Chemistry* 57: 26-37.
- Marmirolo, N., Maestri, E., Gulli, M., Malcevski, A., Peano, C., Bordoni, R. and Bellis, G. D. 2008. Methods for detection of GMOs in food and feed. *Analytical and Bioanalytical Chemistry* 392: 369-384.
- Matsuoka, T., Kuribara, H., Suefuji, S., Miura, H., Kusakabe, Y., Akiyama, H., Goda, Y., Isshiki, K., Toyoda, M. and Hino, A. 2001. A detection method for Recombinant DNA from Genetically Modified Maize CBH351. *Journal of Food Hygienic Society of Japan* 42: 197-201.
- Michelini, E., Simoni, P., Cevenini, L., Mezzanotte, L. and Roda, A. 2008. New trends in bioanalytical tools for the detection of genetically modified organisms: an update. *Analytical and Bioanalytical Chemistry* 392: 355-367.
- Milcamps, A., Rabe, S., Cade, R., De Framond, A. J., Henriksson, P., Kramer, V., Lisboa, D., Pastor-Benito, S., Willits, M. G., Lawrence, D. and Van Den Eede, G. 2009. Validity Assessment of the Detection Method of Maize Event Bt10 through Investigation of its Molecular Structure. *Journal of Agricultural and Food Chemistry* 57: 3156-3163.
- Tavernier, I., Windels, P., Van Bockstaele, E. and De Loose, M. 2001. Use of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products. *European Food Research and Technology* 213: 417-424.
- Terry, C. F. and Harris, N. 2001. Event-specific detection of Roundup Ready Soya using two different real-time PCR detection chemistries. *European Food Research and Technology* 213: 425-431.
- Windels, P., Bertrand, S., Depicker, A., Moens, W., Bockstaele, E. and Loose, M. 2003. Qualitative and event-specific PCR real-time detection methods for StarLink maize. *European Food Research and Technology* 216: 259-263.
- Yang, L., Gua, J., Pan, A., Zhang, H., Zhang, K., Wang, Z. and Zhang, D. 2007. Event-specific quantitative detection of nine genetically modified maize using one novel standard reference molecule. *Journal of Agricultural and Food Chemistry* 55: 15-24.
- Yonemochi, C., Ikeda, T., Harada, C., Kusama, T. and Hanazumi, M. 2003. Influence of transgenic corn (CBH 351, named StarLink) on health condition of dairy cows and transfer of Cry9C protein and cry9C gene to milk, blood, liver and muscle. *Animal Science Journal* 74: 81-88.

Zhang, H., Yang, L., Guo, J., Li, X., Jiang, L. and Zhang, D. 2008. Development of one novel multiple-target plasmid for duplex quantitative PCR analysis of roundup ready soybean. *Journal of Agricultural and Food Chemistry* 56: 5514-5520.