Final Technical Report

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REFINEMENT OF GMO SCREENING METHODS BY COMBINING EXISTING MULTIPLEX PCR APPROACHES WITH LAB-ON-A-CHIP CAPILLARY ELECTROPHORESIS ENDPOINT DETECTION

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Executive Summary

The FSA requires high throughput and low cost screening methods to help Public Analysts provide enforcement of the GM Food and Feed Regulation (EC 1829/20030) for GM food and feed ingredients. British Standard qualitative and quantitative methods (BS EN ISO 21569:2005 and BS EN ISO 21570:2005) are available for GMO testing. These use the polymerase chain reaction (PCR) to target DNA sequences within the GM crops but uptake by enforcement laboratories has been limited due to the nature of the equipment or skill base needed to perform the analyses and produce reliable results.

Project G0322 investigated the use of a lab-on-a-chip capillary electrophoresis technology (Agilent 2100 Bioanalyzer) to deliver simple, cost effective methods for routine DNA analyses. It concentrated on the screening for common markers associated with GM crops, the identification of Roundup Ready soya and the detection of several GM maize varieties.

A novel multiplex GMO screening assay was developed to detect small target sequences (<130bp) found in the Nos terminator, lectin (endogenous soya), zein (endogenous maize), cauliflower mosaic virus (CaMV) and the CaMV 35S promoter. The CaMV target was included in the assay to enable differentiation of samples containing GMO ingredients from those samples which contain material derived from cruciferous plants infected with the CaMV. The assay was applied to certified reference materials, processed ingredients and animal feeds. It was further validated in a trial amongst 10 local authority public analysts. They were provided with a detailed SOP and PCR mastermix reagents and asked to analyze a range of materials. Their results indicated that the multiplex assay worked well with little variation in the number of targets detected by the laboratories for each sample. Results also indicated that it could detect down to 0.1% (w/w) levels of GMO.

A second multiplex assay was developed to detect the endogenous soya lectin gene and targets within the Roundup Ready (RR) soya transgene. This assay was used to estimate the levels of RR soya in some soya based ingredients including soya flour, textured vegetable protein (TVP) and animal

feed. The levels produced compared well with the levels of Roundup Ready soya measured by Real-time PCR. During this study, an automated DNA extraction instrument, Promega's Maxwell 16, was also evaluated. Results indicated that the Maxwell 16 was suitable for reliable extraction of DNA from some food and ingredients but the yield was lower than with other methods.

A third multiplex assay was proposed for the detection of 5 different GM maize varieties (Bt176, Bt11, Mon810, GA21, T25) and endogenous maize gene, zein. This used targets and PCR primer sets from existing published assays that were selected for optimal use on the Bioanalyzer. The individual primer sets were obtained and tested. Results indicated that the individual assays detected the intended GM maize varieties with no cross-reactivity, but when the primers were used together in a multiplex assay there was preferential amplification of some GM maize varieties. Further development and optimisation of the assay is required prior to uptake by food enforcement laboratories.

Glossary

2100 Bioanalyzer : A small-scale capillary electrophoretic system from Agilent Technologies. It uses lab-on-a-chip technology and microfluidics for the specific separation of DNA or protein fragments.

CaMV: Cauliflower mosaic virus. A virus that infects brassica plants.

Capillary electrophoresis (CE) : A method that can be used for DNA fragment separation using small diameter capillary tubes. Fragments are separated based on size and charge and are detected using a variety of methods, in this case laser-induced fluorescence (LIF).

Copy number : The number of copies of a gene per cell. Some genes are multi-copy, i.e. there is more than one copy on the genome.

DNA : Deoxyribonucleic acid. The molecule found in chromosomes that contains the genetic code.

dNTP : Deoxynucleotide-triphosphate. The base units of DNA.

EPSPS : 5-enolpyruvylshikimate-3-phosphate synthase. Enzyme produced from the herbicide tolerant gene found in Roundup Ready Soya.

FluorescenceUnits (FU) : The measure of fluorescence intensity used by the 2100 Bioanalyzer.

Gel electrophoresis : A method used to separate proteins or DNA fragments on acrylamide or agarose gel matrices. Fragments migrate on the basis of size and charge when an electric current is applied. The gel matrix acts as a sieve to separate the fragments based on size.

Gene : An ordered series of nucleotide bases which code for a specific protein.

Genome : The total DNA content of an individual or organelle.

LabChip : Small ($3cm^2$), disposable, single-use plastic and glass units containing etched capillaries attached directly to twelve sample loading wells. DNA fragments up to 1000bp can be separated on the chip by the 2100 Bioanalyser. Currently the Series II LabChips are in use.

PCR : Polymerase Chain Reaction – a method of amplifying a specific gene or region of DNA to produce millions of copies.

Primer : A short oligonucleotide designed to anneal to specific regions of DNA in order to facilitate the PCR. Primers are designed to complement regions of DNA bounding the gene of interest.

***Taq* polymerase :** A specific, heat-stable DNA polymerase used to replicate DNA targets during PCR.

1. Introduction

1.1 Genetically modified foods - labelling legislation and detection

In Europe there is stringent legislation governing the labelling of genetically modified (GM) foods. Regulation 50/2000 established a requirement for labelling of food and food ingredients containing additives and flavourings from GM sources. Regulation 49/2000 introduced a 1% threshold for the adventitious presence of DNA or protein from GM material in conventional food. However, this was altered in Regulation 1829/2003, giving a 0.9% threshold for accidental presence of GM material from approved varieties and a 0.5% threshold for non approved varieties which have received a favourable opinion from the EU Scientific Committee.

Molecular biology methods using the polymerase chain reaction (PCR) have been developed to ensure compliance with the legislation. Many of the earlier methods, covering sampling, DNA extraction, qualitative analysis and quantitative analysis, have been validated and trialled. These have been used to form a series of British Standards (BS EN ISO -21568, -21569, -21570 and -24276)[1].

In the UK, the uptake of GMO analysis methods amongst local authority food enforcement laboratories has been limited as many have lacked the facilities, equipment and skills to get the DNA methods established. However, during 2007, the Food Standards Agency helped 10 laboratories to acquire lab-on-a-chip capillary electrophoresis instrumentation (Agilent 2100 Bioanalyzer). They also funded the development of a number of DNA-based authenticity testing methods which used the Bioanalyzer for end-point detection[2].

1.2 Lab-on-a-chip CE

Lab-on-a-chip capillary electrophoresis (CE) offers distinct advantages for DNA analysis. Rapid fragment sizing and quantification using disposable CE chips enables analysts to remove the uncertainty of performing fragment separation and detection on electrophoresis gels. The CE chips deliver highly reproducible profiles using significantly less sample and “hands-on” time compared to traditional methods. The Agilent 2100 Bioanalyzer uses the DNA 1000 Series II DNA LabChip to detect DNA fragments ranging from 25 to 1000bp and can separate fragments which differ by 5 or more bases. Following analysis a report detailing the fragment size and the concentration of the DNA fragments is produced automatically and the Bioanalyzer software allows efficient comparison

of samples to reference material within a chip or from a previous analysis. This offers significant advantages over traditional gel-electrophoresis endpoint detection.

1.3 GMO analysis & Multiplex PCR

Detection methods use PCR to amplify targets found in the genetically modified plants. The assays target endogenous genes, gene regulatory elements common to a wide range of GMOs and specific targets found within the transgenic DNA. Screening of food and ingredients can be time consuming as analysis is often performed in a sequential manner, analysing for individual targets. Once GM DNA has been identified, real-time PCR is used to quantify the level.

Multiplex PCR can be used to analyse for several DNA targets at the same time. It involves amplification using several sets of primers in a single PCR reaction. As there are an increasing number of GM crop varieties being developed, such approaches will save considerable time and costs by decreasing the number of reactions required to be performed.

Several studies have used multiplex PCR for GMO screening[3] and variety identification[4,5,6,7,8]. Some of these assays were limited to raw materials as the DNA targets were too large to be detected in processed foods where DNA is often highly degraded. Use of the Bioanalyzer for GMO analysis has been evaluated [9,10,11], but there have been no applications to processed food.

In this study we proposed to develop and evaluate three multiplex PCR GMO screening and detection assays for use on the Bioanalyzer. The aim was to develop a series of methods suitable for uptake by UK Public Analysts, adding to their portfolio of Bioanalyzer-based methods.

One of the assays, detecting Roundup Ready soya , had previously been developed by Campden BRI to investigate the effect of processing on the degradation of genetically modified DNA[12,13]. Further validation work and trial by government food enforcement laboratories was proposed.

In order to allow efficient cost effective screening of processed foods and ingredients containing soya and maize, the development and validation of an alternative multiplex PCR GMO screening assay was proposed. Simultaneously targeting small DNA sequences (<130bp) found in the Nos terminator, lectin (endogenous soya), zein (endogenous maize), cauliflower mosaic virus (CaMV) and the CaMV 35S promoter, it would be ideal for screening samples containing degraded DNA. The CaMV target was included in the assay to enable differentiation of samples containing GMO ingredients from those samples which contain material derived from plants naturally infected with the virus. It has been reported that members of the family *Brassicaceae* (*Cruciferae*) as well as *Resedaceae* and *Solanaceae* can potentially be infected[14].

Finally, an attempt to develop a multiplex assay for the detection of GM maize varieties (Mon810, Bt176, Bt11, GA21 and T25) was proposed. It would use existing published primer sequences to establish a method suitable for transfer onto the Bioanalyzer.

2 Methods

All chemicals used for this work, unless otherwise stated, were supplied by Sigma-Aldrich (Poole, Dorset, UK) and were of molecular biology grade or equivalent.

2.1 Genetically modified food materials

2.1.1 Certified reference materials

Roundup Ready® soya (0.1, 0.5, 1.0, 2.0, 5.0 % w/w) and GM maize reference flour materials (5.0 % w/w) for Mon810, Bt176, Bt11, Nk603, GA21 were obtained from Sigma-Aldrich (Poole, Dorset, UK). Certified reference DNA for GM maize T25 was obtained from AOCS Technical Services (P.O. Box 17190, Urbana, USA).

2.1.2 Additional materials

GeMMA proficiency test materials (flour, TVP and animal feed) were obtained from FAPAS (CSL, Sand Hutton, York, UK). Other food and feed materials were obtained from local suppliers.

2.2 Extraction of DNA from samples

2.2.1 CTAB DNA extraction method

Samples (2g) were suspended in 5ml of CTAB buffer (2% CTAB [hexadecyltrimethylammonium bromide], 100mM Tris-HCl, 20mM EDTA, 1.4M NaCl, pH 8.0) and 40µl of Proteinase K solution (20mg/ml) was added. Samples were vortexed vigorously and then incubated overnight at 60^{o}C. After incubation, 1ml of supernatant was removed to a 2.0ml Eppendorf tube, cooled to room temperature and centrifuged at 13,000g for 10 minutes. The clear supernatant was recovered and an equal volume of chloroform added. The solution was vortexed vigorously and then centrifuged at 16,000g for 15 minutes before the upper aqueous layer was removed to a clean 1.5ml Eppendorf tube. An equal volume of isopropanol was added and the DNA precipitated at room temperature for 30 minutes. DNA was pelleted by centrifugation at 16,000g for 15 minutes, washed in 70%v/v ethanol and air dried for 30 minutes at room temperature. The DNA pellet was resuspended in 100µl of ultrapure water and purified using Promega's Wizard® Purification Resin as per the manufacturer's protocol. DNA extracts were recovered in 50µl of 1xTE (10mM Tris-HCl, pH 7.4, 1mM EDTA, pH 8.0) buffer. Final DNA concentrations (ng/µl) were determined by spectrophotometry using a GeneQuant *pro* DNA calculator (Pharmacia, UK).

2.2.2 Promega's Maxwell™ 16 DNA extraction robot

DNA was extracted from the sample (~50mg) using the Maxwell™ 16 DNA extraction robot (Promega Ltd, UK). The Maxwell™ 16 Tissue DNA purification kit (catalogue number AS1030) and associated extraction protocol was used as per the manufacturer's instructions. DNA was eluted in 400µL of elution buffer and the concentration determined by spectrophotometry using the GeneQuant *pro* DNA calculator.

2.3 DNA analysis

2.3.1 PCR

Primers for PCR (Table 1) were obtained from MWG-Biotech UK Ltd (Ebersberg, Germany) and were high-pure salt-free (HPSF) grade. PCR amplification was performed using either a PE9600 or ABI9800 PCR machine (Applied Biosystems, Warrington, Cheshire, UK). The PCR conditions, giving details of reagents and programmes, are found in the individual SOPs in the Appendices.

2.3.2 Conventional gel electrophoresis

PCR products (5µl) were mixed with 1µl loading buffer (10% Ficoll400, 0.25% Bromophenol Blue) and the whole volume loaded onto a 2% agarose gel containing SYBR safe stain. The DNA fragments were separated using 100V for 30 minutes. Gel images were captured using a GelDoc2000 Image Capture System (Bio-Rad, Hemel Hempstead, UK) and Quantity-One software (Vers 4.3.0, Bio-Rad) following the manufacturer's instructions. Hard-copy images of the gels were printed from the system and electronic copies stored in Quantity-One and Tiff file formats.

2.3.3 Capillary electrophoresis using the Agilent 2100 Bioanalyzer for PCR product detection

All PCR products were analysed using Series II DNA 1000 LabChips and the Agilent 2100 Bioanalyzer (Agilent Technologies UK Ltd, Stockport, Cheshire, UK). Reagents were prepared following themanufacturer's instructions. Batches (~500µl) of gel matrix (used to fill LabChip capillaries) were prepared as required or at 4 weekly intervals. All reagents were stored chilled when not in use and allowed to reach room temperature 30 minutes before use. Aliquots (1µL) of the reaction mix were loaded on to the LabChip, as per the manufacturer's instructions, and analysed on the 2100 Bioanalyzer**.**

2.3.4 CaMV sequence analysis and primer design

CaMV sequences were obtained from the GenBank database and aligned using the MegAlign module of LaserGene (Version 5.05) (DNAStar Inc., Madison, WI, USA). Primers were designed using Primer3 design software[15].

Table 1: Primer pairs used in multiplex PCR assay reactions

Target	Primer	Sequence (5' – 3')	Reference
Roundup Ready Soya multiplex PCR assay			
Soya lectin gene	80-for	AAC CGG TAG CGT TGC CAG	Vaïtlingom *et al*. (1999)[16]
	80-rev	AGC CCA TCT GCA AGC CTT T	
EPSPS transgene	117-for	GGT CTA CGA TTT CGA CAG CAC CTT	Garrett *et al*. (2001)[12]
	117-rev	TCA CCG TCT TCC GAT TTC ACC T	
	150-for	ATG ATC GAC GAA TAT CCG ATT CTC	
	150-rev	ATC GCA ATC CAC GCC ATT	
	202-for	CCG CAA ATC CTC TGG CCT T	
	202-rev	GAT GAT CCA GGT GTC GCC TT	
GMO screen multiplex PCR assay			
CaMV 35S promoter	35S-CF3 for	CCA CGT CTT CAA AGC AAG TGG	EN ISO 21569:2005[1]
	35S-CR4 rev	TCC TCT CCA AAT GAA ATG AAC TTC C	
Nos terminator	HA-NOS 118 for	GCA TGA CGT TAT TTA TGA GAT GGG	
	HA-NOS 118 rev	GAC ACC GCG CGC GAT AAT TTA TCC	
Soya lectin gene	STLM-1	AAC CGG TAG CGT TGC CAG	Vaïtlingom *et al*. (1999)[16]
	STLM-2	AGC CCA TCT GCA AGC CTT T	
Maize zein gene	ZET-M1	TGT TAG GCG TCA TCA TCT GTG	
	ZET-M2	TGC AGC AAC TGT TGG CCT TAC	
CaMV	CaMV1156F	AAG CAA AGA CCC TTC GGA GT	This study
	CaMV1659R	CCT TTA GTT GGC TCG AGT AAT CA	
Maize variety multiplex PCR assay			
Mon810	P-E35S for	CAT TTC ATT TGG AGA GGA CAC G	Germini *et al.* (2004)[5]
	MON 810 rev	GCA TTC AGA GAA ACG TGG CAG TA	
Bt11	BT11 for	CTG GGA GGC CAA GGT ATC TAA T	
	BT 11 rev	GCT GCT GTA GCT GGC CTA ATC T	
BT 176	BT176 for	CCC TTC AAC TTC AGC AAC GGC A	
	BT 176 rev	TAG TCG GTC ACG TCG GTC TTC AGG	
GA 21	GA21 for	TCT CCT TGA TGG GCT GCA	
	GA 21 rev	ACG GTG GAA GAG TTC AAT GTA TG	
T25	T25 for	GGC ATG ATG TTG GTT TTT GGC AAA G	Onishi *et al*. (2005)[4]
	T25 rev	AAT TCG AGC TCG GTA CCC CT	
Maize zein gene	ZEIN for	CGC CAG AAA TCG TTT TTC AT	Germini *et al.* (2004)[5]
	ZEIN rev	GGT GGT GTC CTT GCT TCC TA	

3 Results & Discussion

3.1 Multiplex PCR assay for Roundup Ready soya

This multiplex assay had been developed previously by Campden BRI to look at the effect of processing on the detection of genetically modified DNA[12]. Information concerning its development and use are found in Appendix A. Primer concentration had been optimised to obtain four PCR products resolved by the Bioanalyzer which corresponded in size to the lectin gene target of 80bp, and the EPSPS gene targets of 117bp, 150bp and 202bp respectively (Figure 1).

3.1.1 Roundup Ready soya assay validation

The multiplex PCR assay was applied to DNA extracts from RR soya reference materials (0, 0.1, 0.5, 1.0, 2.0, 5 % w/w) prepared using the Maxwell 16 DNA extraction robot. Extraction data from the Maxwell 16 extractions performed on the reference materials and other food materials are found in Appendix B.

Following amplification in the multiplex assay, 1µl of each sample was run on the Bioanalyzer using a Series II DNA 1000 LabChip. The Bioanalyzer software automatically generates a gel-like image showing the amplification product for each sample (Figure 2). It also produces electropherograms and details of the concentration and molarity of each PCR product. The results (Table 2) show that the concentration of the 3 EPSPS amplification products increased with increasing percentage of Roundup Ready Soya . This is particularly evident with the 117bp product which increases from 0.42 ng/µl to 11.27 ng/µl. The 150 and 202bp products were not detected in the 0.1 and 0.5% w/w Roundup Ready soya samples. The amount of lectin product should in theory remain constant, but this is very dependent on the amount and quality of the extracted soya DNA. However, by visualisation of the gel-like image generated by the Bioanalyzer, it is possible to establish if a soya flour sample contains trace amounts, low amounts or high amounts of Roundup Ready soya .

Table 2: PCR product concentration results from the Round up Ready soya assay

Target	Size determined by the Bioanalyzer	Concentration of PCR products (ng/µl) obtained with DNA extracts prepared from the following reference materials (% w/w Roundup Ready soya)					
		0	0.1	0.5	1.0	2.0	5.0
80	88	2.95	2.63	3.71	5.03	4.58	5.68
117	121	N/A	0.42	3.30	7.30	7.89	11.27
150	148	N/A	N/A	N/A	0.17	0.54	1.86
202	205	N/A	N/A	N/A	0.21	0.55	3.27

Direct comparison of concentration of amplified products may be suitable for 100% soya flour but would not be appropriate for estimation of the amount of Roundup Ready soya in a sample that contained other non-soya ingredients. One way to overcome this is to use the ratio of the EPSPS product to the lectin product. This takes into account the type, quality and concentration of DNA produced from the sample.

Replicate analyses were carried out on 0.1%, 1% and 5% w/w reference materials and ratios of the 117bp EPSPS product to the 80bp lectin product were plotted (Figure 3A). In order to achieve a linear regression between the ratios a log scale % Roundup ready axis was used (Figure 3B).

The multiplex assay was used to assess the levels of Roundup Ready soya in three different sample types: soya flour, TVP and animal feed (Table 3). These samples were obtained from FAPAS for use in proficiency test rounds where the levels of Roundup Ready soya are determined by participants using real-time PCR. The 0.1% , 1.0% and 5.0 % w/w reference materials were also analysed at the same time and the ratio of the 117/80bp target determined. Calibration curves were constructed and the amount of Roundup Ready soya determined. The analysis was carried out on two different days using different DNA extracts and replicate PCR reactions.

Table 3: Analysis of food and feed samples

Sample	Analysis performed on the Bioanalyzer		Real-Time PCR
	Mean % w/w RR soya determined (n=3) Day 1	Mean % w/w RR soya determined (n=3) Day 2	GeMMA assigned value for % w/w RR soya
Soya Flour	2.84	2.33	2.0
TVP	0.28	0.35	0.21
Animal Feed	11.42	7.05	5.1

The results show that the assay produces results which have a reasonable correlation with the GeMMA assigned value, although there appears to be an over estimation of the % RR soya. This could be due to inaccuracies of PCR fragment concentration determination on the Bioanalyzer and the measurement of PCR products after 40 cycles of PCR.

3.1.2 Transfer of the Roundup Ready soya assay to a Public Analyst laboratory

The Multiplex PCR assay for Roundup Ready soya was used by Somerset Scientific Services for semi-quantitative analysis. Assay details were provided and all reagents including primers were obtained by the laboratory. The laboratory also used its own in-house DNA extraction procedure. They established that the assay worked on certified soya reference materials and then applied it to a range GeMMA proficiency test materials. These materials included soya flour, wheat flour containing soya and a baked product.

They performed the analysis on the samples and also included 0.5%, 2% and 5% reference materials, producing calibration curves from the ratio of the lectin and two EPSPS PCR products. A summary of the results is found in Table 4. Analysis of the same 1.0 % RR soya control material was also carried out for quality control purposes. The lectin/116 ratios gave a value of 1.3 % with a standard deviation of 0.58 from 30 separate analyses. The lectin/196 ratios gave a value of 1.05 % with a standard deviation of 0.55 from separate 38 analyses. Their results from the GeMMA rounds were satisfactory and indicated that the method was suitable for semi-quantitative analysis for Roundup Ready soya.

A presentation given by Somerset Scientific Services covering their use of the Roundup Ready Soya assay is found in Appendix C. The presentation was given as part of a DNA methods/Bioanalyzer training course held at Campden BRI during April 2007.

Table 4: Results obtained by Somerset Scientific Services using the Roundup Ready soya assay

Sample type	**Somerset result obtained with assay** **(% w/w GM soya in total soya)**	**GeMMA assigned value determined by Real-time PCR** **(% GM w/w GM soya in total soya)**
Soya flour in wheat flour	3.5	2.9
Soya flour	3.5	2.7
Mixed flours	>2.0	5.3
Baked product	2.1	1.6
Soya flour	2.2	2.7
Mixed flours	>2.0	2.7

3.2 Development of a multiplex PCR GMO screening assay

3.2.1 Choice of primers

Primer sets detailed in British Standard (BS EN ISO) 21569:2005 and published papers were assessed for their use in the detection of CaMV 35S promoter, Nos terminator, endogenous maize and endogenous soya DNAs. The criteria for selection was amplicon size, annealing conditions and the ability to be resolved on the Bioanalyzer. Primer sets with an amplicon length <150 bp were chosen to enable amplification of target sequences in processed foods and ingredients. Details of the primers selected are found in Table 1.

3. 2. 1. 1 CaMV primer design

A total of 20 complete or partial DNA sequences from the cauliflower mosaic virus (CaMV) were recovered from the GenBank database and aligned using the MegAlign software. A consensus sequence was produced from these aligned sequences (Figure 4). A 688bp region from the 5' end of the CaMV consensus sequence was used to design PCR primers. This region of the CaMV was selected as it did not include any part of the 35S gene, and therefore reduced the risk of identifying the existing 35S target.

Conditions for primer design were restricted to producing PCR product sizes within a 50bp to 150bp length limit. A total of fifty theoretical primer pairs were identified by Primer3. After manual assessment of these primer sets, a total of four forward primers and three reverse primers were identified for further investigation. BLAST searches against GenBank were performed using the remaining seven primers to ensure that they did not show high homology with other species. No significant levels of homology were shown with any of the primers. All combinations of the four forward and three reverse primers were used to amplify DNA from CaMV material. Primer combinations were assessed for amount of PCR product and for the production of a single PCR product of the expected size. Gel images from primers giving 89, 114, 104 and 79bp products with CaMV DNA are found in Figure 5. All apart from the 114bp primer set gave a good yield of PCR, product although there were some further non-specific bands with Roundup Ready soya DNA for the 89 and 79bp primers. Further cross-reactivity studies for 89 and 104bp primers are found in Figure 6. The primers giving the 104bp product gave greatest yield of PCR product and did not cross react with Roundup Ready soya , maize, wheat and other cereal DNAs. This primer set (Table 1) was subsequently used for further development of the multiplex GMO screening assay.

3.2.2 Evaluation of GMO screening primers

Each primer set was applied to DNA prepared from 5% Certified Reference Materials to ensure that they were amplifying the appropriate amplicon (data not shown). The 5 primer sets were then incorporated into a multiplex assay. Initial efforts to combine the primers in a single assay resulted in unequal amplification of the individual targets. This was overcome by use of a commercial Multiplex PCR master mix kit from Qiagen. This mastermix contained pre-optimised levels of Hot Start Taq, $MgCl_2$, dNTPs and novel factors for amplification of several DNA targets at the same time. Initial results from the optimised multiplex assay when applied to individual GM variety reference DNAs are shown in Figure 7. The assay was applied to 10^{-1} and 10^{-2} dilutions of DNA extracts prepared from 5% GM varieties. For Roundup Ready soya , the 35S promoter target, Nos terminator target and the endogenous soya (lectin gene) target were amplified. The 35S promoter, Nos terminator and endogenous maize (zein) were amplified for the GM maize varieties NK603 and Bt 11 DNA. Only 35S promoter and zein were amplified when Mon810 and Bt176 DNA were used and only the Nos terminator and zein targets were amplified with GA 21 DNA. The multiplex assay was also applied to DNA extracts prepared from wheat flour and no amplification products were observed (data not shown).

Application of the assay to dilutions of Roundup Ready Soya and NK603 DNA (Figure 8) showed that amplification of the 35S and Nos targets was achieved in a 10^{-4} dilution of the extracted DNA. The amount of extracted DNA estimated to be present in the 10^{-1} reaction was 30ng. As the DNA was extracted from a 5% (w/w) GM variety flour the amount of GM DNA is estimated to be 1.5ng. In the 10^{-4} dilution approximately 1.4 pg of GM DNA would be present. For Roundup Ready soya this represents <10 genome equivalents. The lectin and the zein targets were amplified from the 10^{-5} dilution of the RR soya and NK603 maize respectively. When the multiplex assay was applied to DNA extracts prepared from plant material containing the CaMV virus, both the 35S target and the CaMV were amplified in the 10^{-5} dilution. As this DNA contains both plant and viral DNA, the actual level of CaMV DNA in this dilution is unknown.

The results show that the multiplex PCR GMO screening assay can detect low quantities of GM DNA and this makes it suitable for use on most food ingredients and products which contain soya and maize.

3.2.3 GMO screening assay - Application to TVP and animal feed samples

The multiplex assay was applied to several TVP and animal feed samples obtained from GeMMA proficiency test rounds and from in-house work (Figure 9).

TVP sample SP01A, which was prepared from a commercial source of material, was supplied as being free from Roundup Ready Soya[17]. The multiplex assay gave a PCR product for lectin, but also gave zein and Nos terminator products in the reaction performed on the 1/10 DNA dilution, suggesting possible presence of trace levels of GM maize DNA. Interestingly, some laboratories that participated in the SP01 GeMMA round also identified the presence of a Nos product in this sample[17].

TVP sample SP01B, prepared from the same source material at SP01A, contained approximately 5% Roundup Ready Soya[17] and the multiplex assay gave lectin, 35S promoter and Nos terminator products. It also gave a low level of zein product, again indicating that the sample contained trace levels of maize.

The GeMMA animal feed sample MP02 contained Roundup Ready Soya at approximately 3 % w/w with respect to total soya and low levels of non-GM maize[18]. The multiplex assay gave products for lectin, zein, 35S and Nos.

The other commercial animal feed samples (1-4) had been previously analyzed by Campden BRI using a Roundup Ready Soya specific assay and were found to contain between 0.5 and 2% w/w. The multiplex assay gave products for lectin, zein, 35S and Nos with three of the samples and lectin, 35S and Nos with the other sample. This latter sample had a formulation without maize.

The results show that the GMO multiplex assay works well on processed TVP and animal feeds.

3.2.4 GMO screening assay - Method trial by government enforcement laboratories

The Multiplex GMO screening assay was evaluated in a trial amongst nine local authority Public Analyst laboratories and the Laboratory of the Government Chemist (10 laboratories in total). All participating laboratories owned an Agilent Bioanalyzer and had received some training from Campden BRI in DNA methods for food authenticity in the past. They were supplied with an SOP, mastermix reagents (primer mastermix and Qiagen Mutliplex PCR kit reagent), two controls and six blind samples. They were asked to extract the samples using their own standard method, dilute the extracts 1/5 and 1/25 (or prepare DNA concentrations of 50ng/µl and 10ng/µl), perform PCR and report details of the amplification products detected. The compiled results for all the laboratories are found in Appendix D. Table 5 lists the controls and blind samples and also summarises the results returned.

The results from the analysis of the 0.1% Bt 11 Maize (sample A) indicate that the 35S/Nos target was detected but was dependent on the concentration of total DNA used in the assay. Only one of the laboratories failed to detect the target in the 1/5 dilution but three laboratories failed to detect it in the 1/25 dilution. All the laboratories detected the zein and 35S/Nos targets in the 1/25 dilution from 1% Bt11 Maize flour (sample D). Interestingly, two laboratories failed to detect the 35S/Nos target in the 1/5 dilution, suggesting that PCR inhibition may have occurred. The endogenous gene target zein was detected by all laboratories in the 1/5 and 1/25 dilutions of the maize containing samples (A,D, E and F). It was also detected by 5 laboratories in the 1/5 dilution of the TVP (sample C). Only one laboratory detected it in the 1/25 dilution, suggesting that only trace amounts of maize was present.

In the processed matrices, TVP (sample C) and animal feed (sample F), the 35S/Nos target was detected by all the laboratories in both the 1/5 and 1/25 dilutions. These samples contained Roundup Ready Soya soya but also contained low levels of maize.

In the sample containing CaMV (sample B) the CaMV and 35S/Nos targets were detected by all laboratories in the 1/5 and 1/25 dilutions. Three laboratories also detected the zein target, again possibly suggesting that a trace amount of maize was present.

Although the assay appears sensitive enough for low level detection (0.1%w/w) of GM soya and maize in flours and some processed materials, it has not been tested extensively on complex food products containing a range of ingredients. In order to achieve amplification of the 35S/Nos target in such samples, it might be appropriate to increase the number of PCR cycles and/or use a higher level of extracted DNA. Forty PCR cycles were specified in the trial method but this could be increased to 45 or even 50 cycles if required. Similarly only 2µl of DNA extract was used in the PCR

reaction; again, this could be increased to 5µl for samples where soya and maize is a minor ingredient. It would therefore be recommended that laboratories interested in taking up the method conduct further validation studies on a range of sample types to demonstrate that it is fit for purpose.

Feedback from the inter-laboratory trial was generally very good with several laboratories indicating that the SOP and assay were straightforward and the results easy to interpret. One laboratory gave the following comment:

"Many thanks for organising such a useful exercise. Considering that we are looking at an end-point detection system with a multiplex of 5 primer pairs, I was very pleased with the assay, and commend CCFRA in the easily understood protocol and the way the PCR has been optimised."

This multiplex PCR GMO screening method offers a significant benefit to laboratories working on limited financial and time resources, who lack real-time PCR and the capability for high through-put analyses. It can be used as a preliminary screen to investigate samples for GM DNA and help spot false positives for the 35S promoter target. It would be expected that analysts perform further tests on samples where the 35S/Nos target is detected. Identification and quantification of GM varieties is required for enforcement of GMO labelling legislation.

Table 5: GMO screen trial summary results from the 10 Public Analyst Laboratories

Sample	Target (bold indicates target present in sample)	No. of Laboratories detecting the target at...	
		1/5[1]Dilution	1/25Dilution
A.Maize flour (0.1% Bt11 maize)	**Zein**	9[2]	10
	lectin	0	0
	CaMV	0	0
	35s/Nos	8[2]	7
B. Flavour mix (CaMV +ve)	Zein	3	3
	lectin	0	0
	CaMV	9	10
	35s/Nos	9	10
C .TVP (3%RR soya)	Zein	5	1
	lectin	9	10
	CaMV	0	0
	35s/Nos	9	10
D. Maize flour (1% Bt 11 maize)	**Zein**	9	10
	lectin	0	0
	CaMV	0	0
	35s/Nos	7	10
E. Maize polenta (no GM)	**Zein**	9	10
	lectin	0	0
	CaMV	0	0
	35s/Nos	1	0
F. Animal Feed	**Zein**	9	9
	lectin	8	10
	CaMV	0	0
	35s/Nos	9	10
GM soya/GM maize/CAMV DNA control	**Zein**	9	10
	lectin	9	10
	CaMV	9	10
	35s/Nos	9	10
0.5%GM soya reference flour control	Zein	1	0
	lectin	9	10
	CaMV	0	0
	35s/Nos	9	10

[1] only 9 laboratories submitted results data for the 1/5 dilution

[2] one laboratory had problems with 1/5 dilution analysis but indicated that the lectin and 35S/Nos products were detected

3.3 GM Maize identification

3.3.1 Development of multiplex PCR assay

Published primer sets[3, 4, 5, 6, 7 , 8] for the detection of GM maize varieties were reviewed and suitable ones were selected for the detection of Mon 810, Bt 11, Bt 176, GA21, T25 and the maize zein gene. The amplicon sizes for each of these ranged from 110 to 311bp (Table 6). All the primers sets were suitable for use in a PCR reaction with an annealing temperature of 60^{o}C .

The primers were obtained and tested individually on DNA extracts prepared from certified reference materials. They were also tested for cross-reactivity with other GM maize varieties. Results from PCR reactions with Mon 810, Bt 11, Bt 176, GA21 and zein primers are shown in Figure 10. The results for the T25 primer set are not shown.

All primer sets amplified the DNA from their respective GM varieties. The size of each amplification product was determined using the Bioanalyzer (Table 6). The results show that the fragments could easily be resolved on the DNA 1000 labchip. The zein primer set produced single PCR products of about 150 bp with Bt 11, GA 21 and T25, but a second product of about 169 bp was obtained with Bt 176 and Mon 810 maize varieties. This was not reported by the original authors of the paper from which the zein primer set was obtained[5]. Further investigation was not pursued during this project, as the double zein PCR product did not interfere with the detection of other GM maize targets.

Table 6: Details of amplicon sizes for the GM maize assay

Primer set	Expected size of product (bp)	PCR product size determined using Bioanalyzer (bp)
Mon 810	110	117
Bt 176	209	214
Bt 11	189	192
GA 21	270	267
T25	311	315
Maize (zein)	139	150 (all varieties) 169 (additional fragment obtained with Bt176 &Mon 810)

The GM maize variety primers were combined in a multiplex assay and applied to 10^{-1} and 10^{-2} dilutions of both individual GM maize variety DNAs prepared from 5% CRMs and also to a GM maize variety DNA admixture serially diluted to 10^{-5}.

Variety specific and zein PCR products of the anticipated size were obtained from the individual Bt176, Bt11, GA21 and T25 DNAs but no variety specific amplification product was observed with Mon810 (Figure 11). When the multiplex assay was applied to the variety DNA mixture, no amplification product was produced from the Mon810 primers (Figure 12). It was also noted that amplification products from Bt11, Bt176 and GA21 were observed for all DNA dilutions, whereas the T25 product was only detected in the 10^{-1} and 10^{-2} dilutions. This suggests that amplification is not equal for all primer sets, particularly with respect to the Mon810 set, and that further optimisation to establish appropriate primer concentrations is required.

Nonetheless, these results have shown that it is feasible to use a multiplex PCR assay to identify GM maize varieties and that by using the Bioanalyzer, easy identification of GM Maize variety PCR products can be achieved. Over 25 GM maize varieties have been developed for commercial use around the world. Development of a series of multiplex assays to detect 5 or more varieties at a time will significantly reduce the time and cost needed for analysis.

4 Future Work

This series of multiplex PCR assays has been developed for specific use by UK local government enforcement laboratories to help them ensure that food operators are complying with GMO labelling legislation. The assays only cover GM soya and a limited number of GM maize varieties; therefore, there is a significant need for development of similar assays to detect other approved and non-approved GM maize varieties. The GM maize variety assay was developed to detect 5 varieties. It may be possible to extend the numbers of varieties in a single multiplex assay but this may lead to lower sensitivities for detecting some of the varieties. However, it is possible to envisage development of a series of GM maize multiplex assays with separate assays for those varieties which are approved and others for the non-approved varieties. Similar assays could also be developed to screen for GM markers found in other commodity crops such as rice, which has recently been the focus of attention when a non-approved GM variety, Bt63, was found in imported rice products.

5 Acknowledgments

The financial support of the Food Standards Agency is gratefully acknowledged.

Campden BRI thanks Agilent Technologies (Tobias Preckle, Rainer Nitsche and Mark Jensen) and Promega Life Sciences (Stephen Orr) for support and useful discussions concerning the 2100 Bioanalyzer and Maxwell 16 respectively.

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Lancashire County Analysts Laboratory

City of Edinburgh Council

Leicestershire Scientific Services

Durham Scientific Services

Worcestershire Scientific Services

Hampshire Scientific Services

Cardiff Scientific Services

Minton, Treharne & Davies Ltd

Laboratory of the Government Chemist

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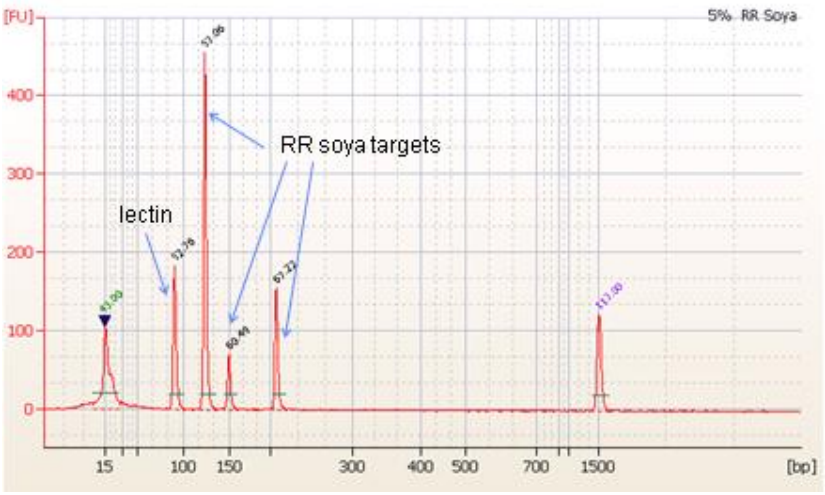
7 Figures

Figure 1

Roundup Ready Soya Multiplex PCR assay products.



An electropherogram image generated by the Bioanalyzer from an amplification of DNA extracted from the 5% (w/w) Roundup Ready Soya certified reference material. Four amplification products are visible: the endogenous soya lectin product and three products from amplification of Roundup Ready Soya transgene (EPSPS) DNA.

Figure 2

Application of the Roundup Ready Soya Multiplex PCR assay to certified reference materials

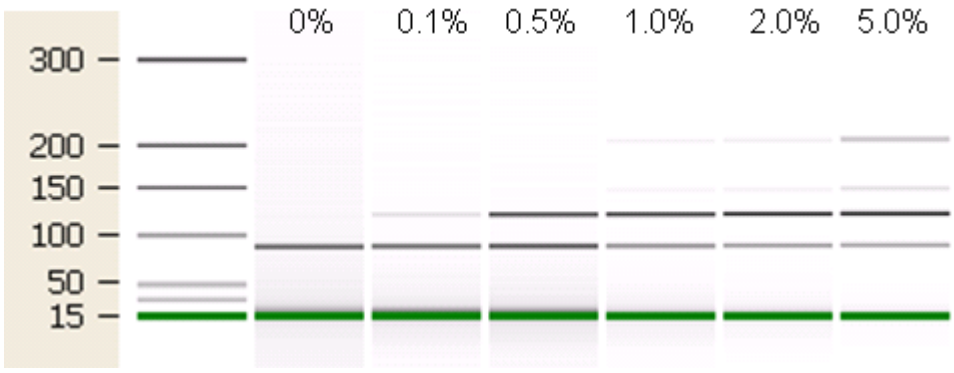
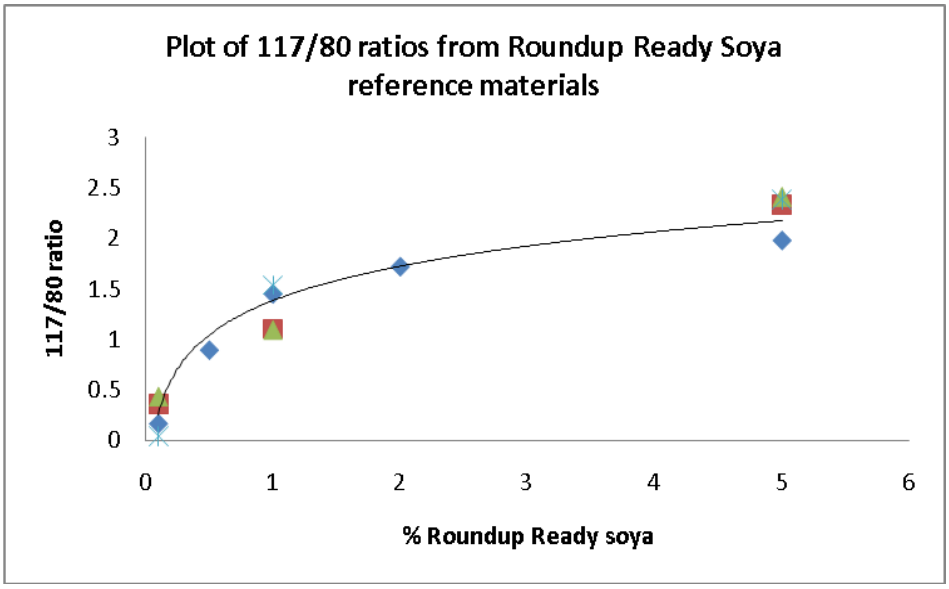


Figure shows a gel-like image generated by the Bioanalyzer. It shows PCR products from certified reference materials (0-5%w/w) and a DNA ladder (bp). EPSPS products increase with increasing levels of Roundup Ready Soya.

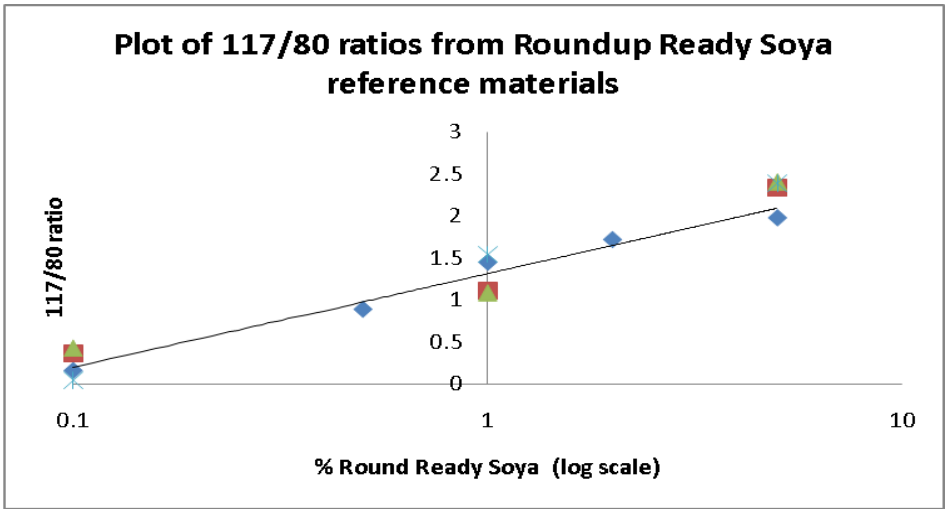
Figure 3

Calibration curves for the Roundup Ready Soya Multiplex PCR assay

A



B



These graphs show 117/80bp ratio data produced from the analysis of 0.1%, 1.0% & 5% Roundup Ready certified reference materials analysed on four separate occasions. The 0.5% and 2% references have been analysed on one occasion.

A. Shows the 117/80 ratios reaching a plateau with higher levels of Roundup Ready Soya

B. A linear relationship can be achieved using a log scale on the % Roundup Ready Soya axis.

Figure 4

CaMV sequence showing primer sites

TAATACTGAAAATGAATTAGCTAGGGTAAGCCAAAACATAGATCTTTTAAAGAATAAATTAAAAGAAATCTGT
GGAGAATAAAATGAGCATTACGGGTCAACCGCATGTTTATAAAAAAGATACTATTATTAGACTAAAACCATTG
TCTCTTAATAGTAATAATAGAAGTTATGTTTTTAGTTCCTCAAAAGGGAACATTCAAAATATAATTAATCATCTT
AACAACCTCAATGAGATTGTAGGAAGAAGCTTACTCGGAATATGGAAGATCAACTCATACTT**CGGACTAAGC**
AAAGACCCTTCGGAGT*CCAAATCAAAAAACCCGTCAGTTTTTAATACTGCAA**AAACCATTTTTAAGAGTGGG**
GGGGT**TGATTACTCGAGCCAACTAAAGG**AAATAAAATCCCTTTTAGAAGCTCAAAATACTAGAATTAAAAGTC
TAGAAAAAGCAATTCAATCCTTAGATAATAAGATTGAACCAGAGCCCTTAACTAAAGAAGAAGTTAAAGAGCT
AAAAGAAT**CGATTAACTCGATCAAAGAAGGA**TTAAAGAATATTATTGGCTGAAATGGCTAATCTTAATCAAAT
CCAAAAAGAAGTCTCTGAAATCCTCAGTG**ACCAAAAATCCATGAAAGCG**GATATAAAAGCTATCTTAGAATTA
TTAGGATCCCAAAATCCTATTAAAGAAAG

Sequence of the 5' region of the CaMV genome used for primer design. Primers are shown under lined.

*The region CGGACTAAGCAAAGACCCTTCGGAGT encompasses three overlapping primers which are not differentiated in this figure.

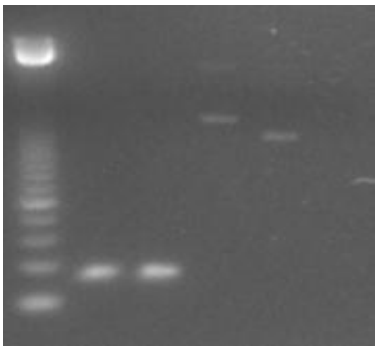


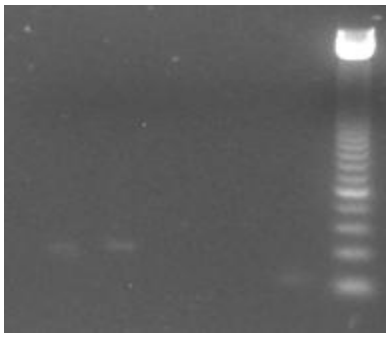
Figure 5

Electrophoresis gel images showing PCR products from evaluation of 4 different CaMV primer sets

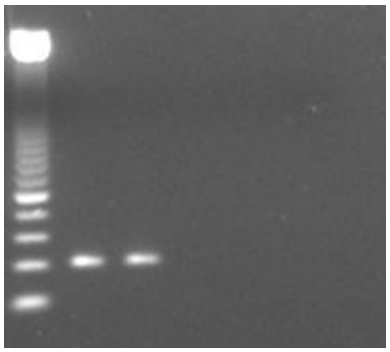
Key

CaMV DNA(1&2), Roundup Ready Soya DNA (3&4) and a water control (5). L - 50bp ladder.

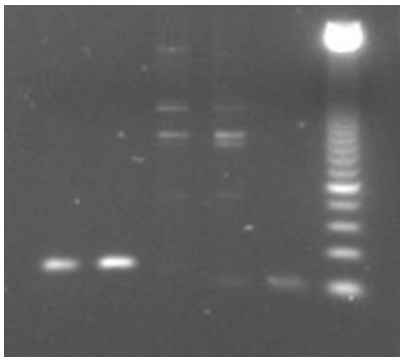
A. L 1 2 3 4 5 **89bp CaMV Product**



B. 1 2 3 4 5 L **114bp CaMV Product**



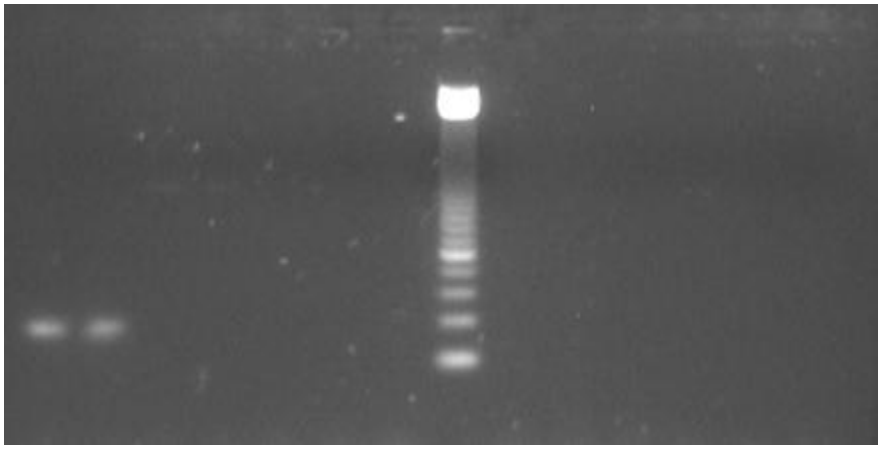
C. L 1 2 3 4 5 **104bp CaMV Product**



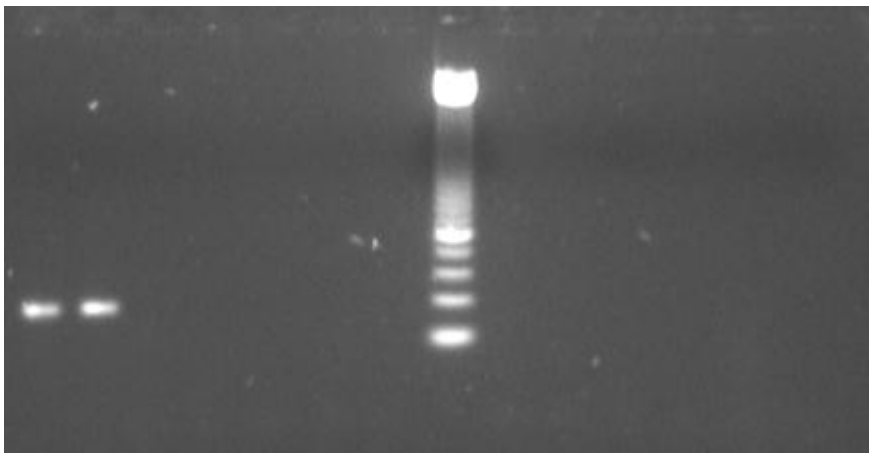
D. 1 2 3 4 5 L **79bp CaMV Product**

Figure 6

Electrophoresis gel images of amplification products from cross reactivity studies for two CaMV primers sets (89 bp & 104bp CaMV products)



A. 1 2 3 4 5 6 7 L 8 9 10 11 12 13 **89bp**



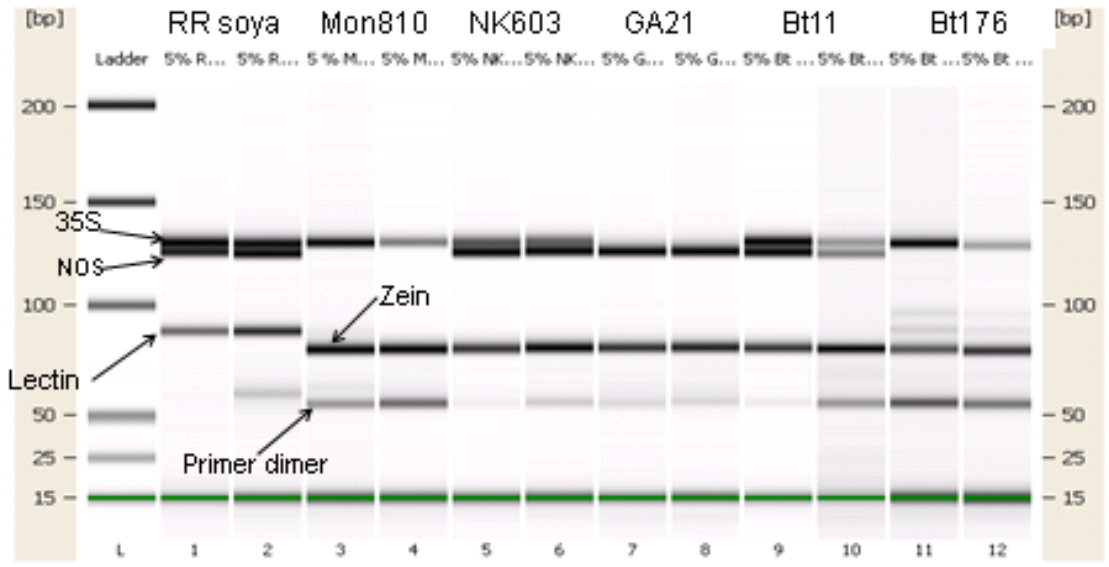
B. 1 2 3 4 5 6 7 L 8 9 10 11 12 13 **104bp**

Key

1&2-CaMV DNA, 3&4- Roundup Ready Soya DNA, 5&6- Maize DNA. 7&8- Wheat DNA, 9&10- Cereal DNA, 11&12- Cornflakes DNA, 13- H_2O control. L-50bp ladder.

Figure 7

Application of Multiplex PCR GMO screening assay to certified reference materials



The assay was applied to 10^{-1} and 10^{-2} dilutions of DNA extracts prepared from 5% (w/w) reference materials.

Figure 8

Determination of sensitivity Multiplex PCR GMO screening assay

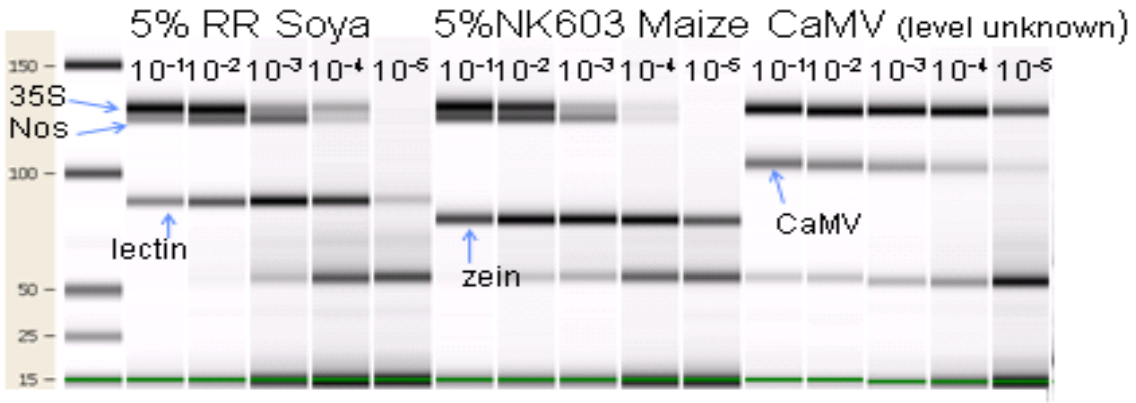


Figure 9

Application of Multiplex PCR GMO screening assay to TVP and animal feed samples

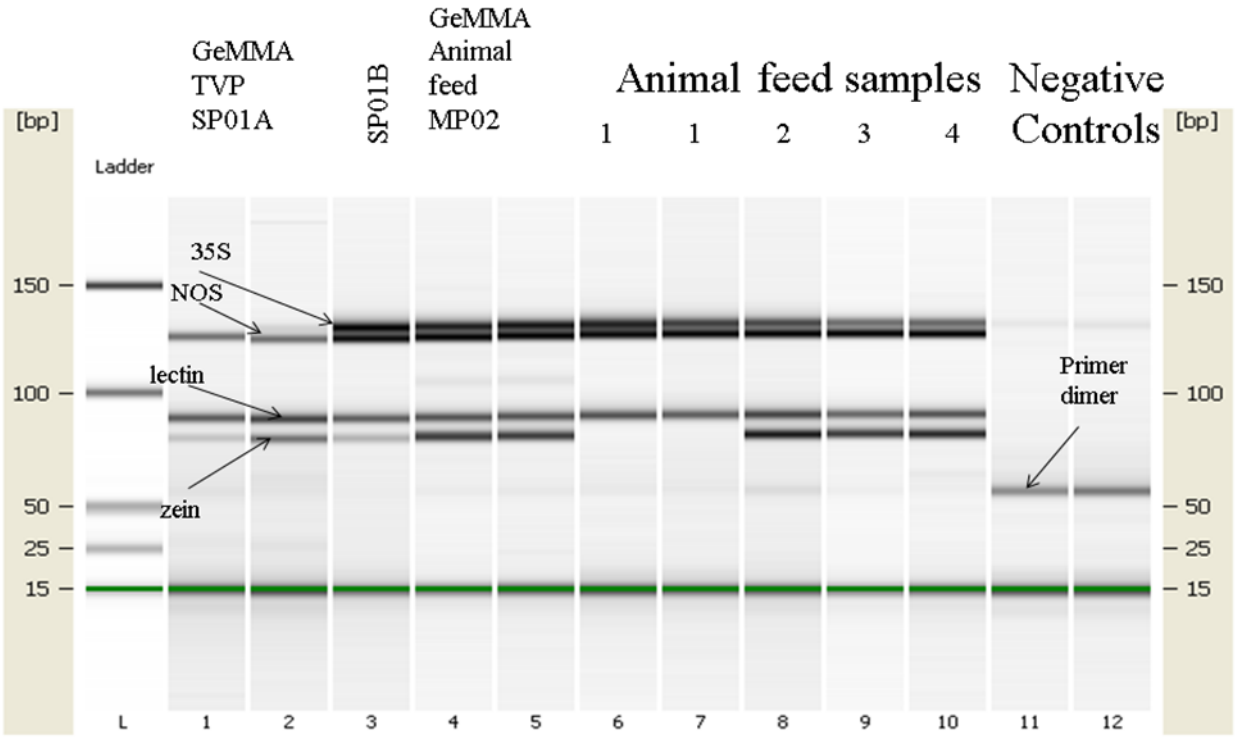
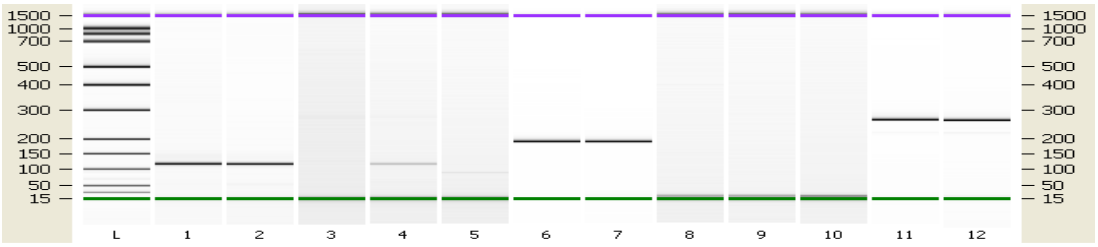
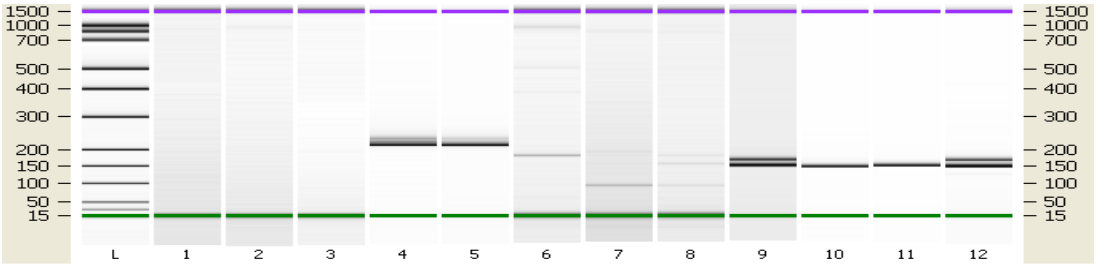


Figure 10

Bioanalyzer image showing PCR products obtained with GM maize variety primers



1	Mon 810 DNA 1/10 dilution Mon810 primers	7	Bt11 DNA 1/100 dilution Bt11 primers
2	Mon 810 DNA 1/100 dilution Mon810 primers	8	GA21 DNA 1/10 dilution Bt11 primers
3	Bt11 DNA 1/10 dilution Mon810 primers	9	Mon810 DNA 1/10 dilution Bt11 primers
4	GA21 DNA 1/10 dilution Mon810 primers	10	Bt176 DNA 1/10 dilution Bt11 primers
5	Bt176 DNA 1/10 dilution Mon810 primers	11	GA21 DNA 1/10 dilution GA21 primers
6	Bt11 DNA 1/10 dilution Bt11 primers	12	GA21 DNA 1/100 dilution GA21 primers



1	Bt11 DNA 1/10 dilution GA21 primers	7	GA21 DNA 1/10 dilution Bt176 primers
2	Mon810 DNA 1/100 dilution GA21 primers	8	Mon810 DNA 1/10 dilution Bt11 primers
3	Bt176 DNA 1/10 dilution GA21 primers	9	Bt176 DNA 1/10 dilution zein primers
4	BT176 DNA 1/10 dilution Bt176 primers	10	GA21 DNA 1/10 dilution zein primers
5	Bt176 DNA 1/100 dilution Bt176 primers	11	Bt11 DNA 1/10 dilution zein primers
6	BT11 DNA 1/10 dilution Bt176 primers	12	Mon810 DNA 1/100 dilution zein primers

Bioanalyzer generated gel images of PCR products. The PCR reactions were carried out on dilutions (1/10 and /or 1/100) of DNA extracted from certified reference materials.

Figure 11

GM maize variety multiplex assay applied to individual GM maize variety DNA

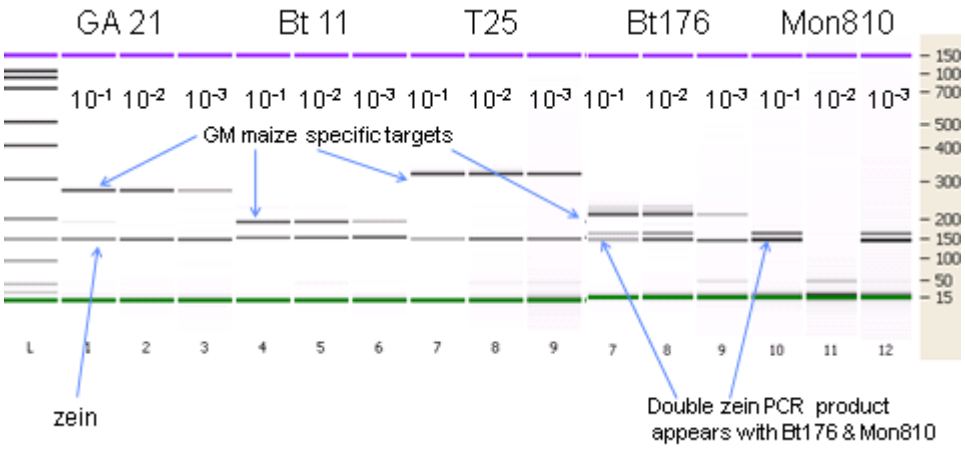
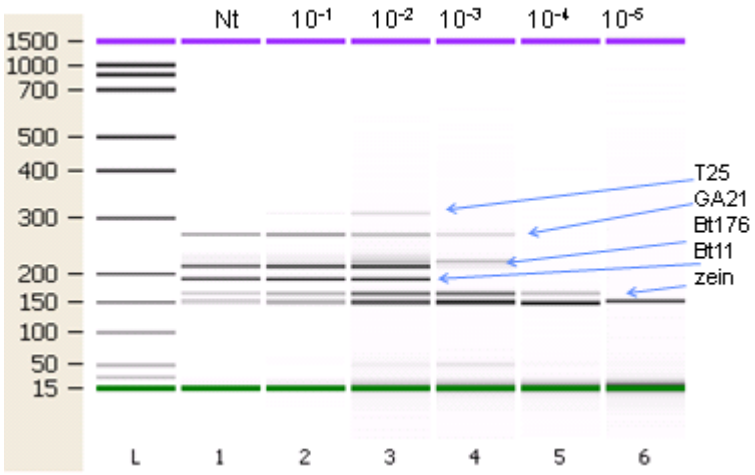


Figure 12

GM maize variety multiplex assay applied to individual GM maize variety DNA



8 Appendix

A. Information from a CCFRA report[12] concerning the development of the Roundup Ready Soya multiplex PCR assay

B. DNA extraction data obtained using the Maxwell 16

C. Presentation by Somerset Scientific Services covering use of the Roundup Ready Soya multiplex PCR assay

D. Results from the Public Analysts' trial of the GMO screening method

E. Standard Operating Procedure for the detection of Roundup Ready soya using the CCFRA multiplex PCR Assay

F. Standard Operating Procedure for the simultaneous detection of general DNA targets associated with presence of genetically modified soya and maize

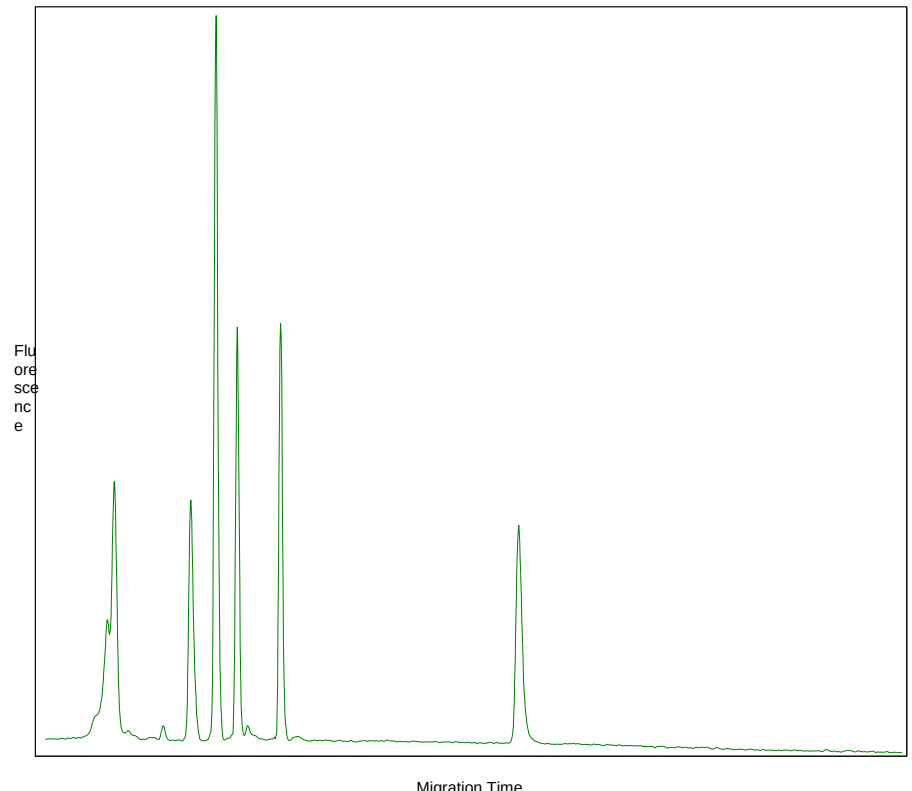
A. Information taken from a CCFRA report[12] concerning the Roundup Ready Soya Multiplex PCR Assay

Development of a model assay system to assess effects of processing on the detection of Roundup Ready soya DNA.

The aim was to develop a model assay that could be used to assess the quality of DNA extracted from heat processed soya flour samples. In particular it could be used to investigate differences in PCR amplification between small DNA targets. A single multiplex PCR assay was developed that enabled four RR soya targets to be analysed in a single reaction mix. Primer concentration was optimised in order to obtain four PCR products resolved by gel-electrophoresis which corresponded in size to the lectin gene target of 80bp, and the EPSPS gene targets of 117bp, 150bp and 202bp respectively.

Although gel-based analysis enables sizing of PCR products, it cannot be used to give accurate information on the quantity of a PCR product. Therefore, post–PCR analysis was performed using the Agilent 2100 Bioanalyser, which can accurately size and quantify PCR products. Initially this was carried out using the DNA 7500 LabChip®. Four peaks were observed, corresponding to the PCR products within the lectin and EPSPS genes. However, the 80bp peak from the lectin gene was not completely resolved from the alignment marker so quantification was not possible. Subsequently, post-PCR analysis was performed using the DNA 500 Labchip® when it was made available and resolution of all four peaks was observed (Figure 1).

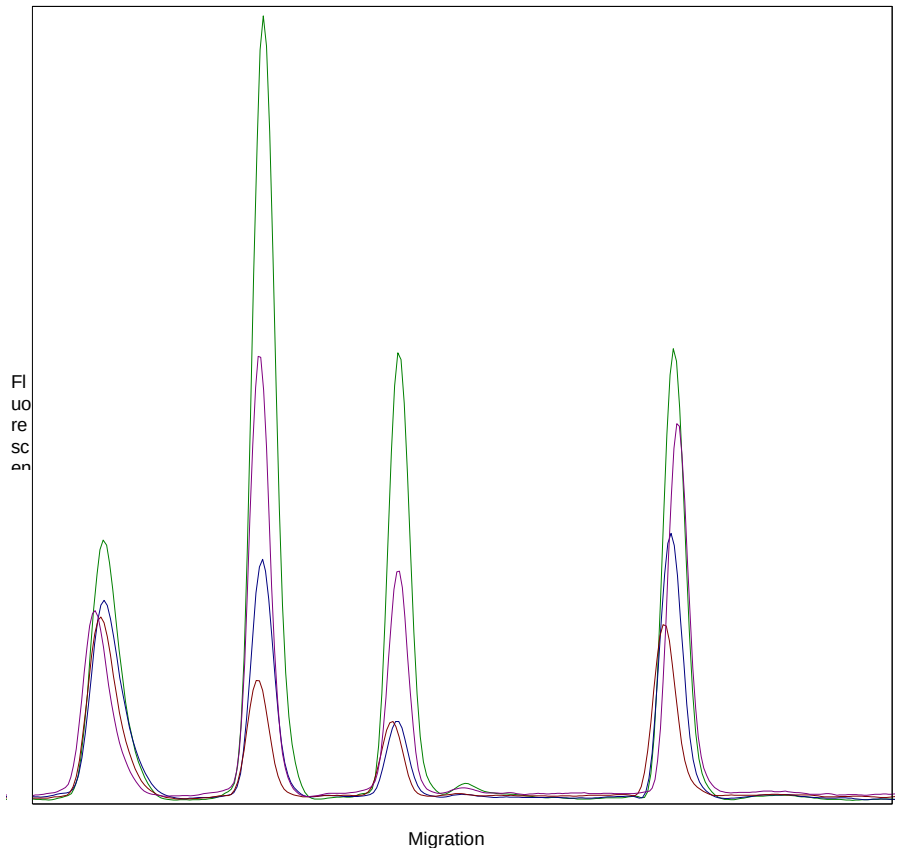
Figure 1: Multiplex assay for RR soya. Peaks produced by the four PCR products when analysed with the Agilent 2100 Bioanalyser and DNA 500 Labchip®



A. Information taken from a CCFRA report[12] concerning the Roundup Ready Soya Multiplex PCR Assay

The multiplex PCR assay was applied to DNA from the RR soya flour reference materials. (Figure 2). The results show that there is an increase in the peak heights of the 117bp, 150bp and 202bp products and no change in peak height in the 80bp product. The increase in peak heights of the 3 products from the EPSPS gene corresponds to the increase in RR content of the soya flour. No increase in the peak height of the product from the lectin gene was expected, as it is common to both the GM- and non-GM soya. It should, therefore, be possible to estimate levels of GM soya in an unknown material by applying the multiplex assay and comparing the peak heights from the PCR products with those produced from reference materials. The assays would have to be performed using a limited number of PCR cycles in order to perform the end-point detection during linear stages of amplification. The reference materials and the unknown samples would also have to be similar in nature.

Figure 2: Peaks produced by the Agilent 2100 Bioanalyser using the RR multiplex assay on soya DNA samples containing different levels of RR soya



A. Information taken from a CCFRA report[12] concerning the Roundup Ready Soya Multiplex PCR Assay

The effect of heating time and pH on detection and quantification of GM-DNA

The multiplex PCR assay was applied to soya flour samples boiled at either pH 3.3, 4.3 or 6.7 for up to 21 minutes. The resulting PCR products were analysed using the DNA 7500 Labchip. Comparison of PCR products produced after heating for various times at pH 3.3 and 6.7 is shown in Figure 7. At pH 3.3 after 3 minutes the peak heights for the 150bp and 202bp products decreased, and after 6 minutes very few products were observed. This differed from heating at pH 6.7 where no marked differences in peak height were observed. For accurate determination of the quantity of each PCR product, the samples were applied to the DNA 500 Labchip. The concentration of each PCR product was calculated using the Agilent software. At pH 3.3 where an effect of heating time was observed, the amount of each PCR product at each time point was compared to the amount of each product at 0 minutes (Table 1). At pH 3.3, the relative amount of the 80bp product was reduced to 48% after 15 minutes and no product was detected at 18 or 21 minutes. After 15 minutes, the relative amounts of products of 118bp and 145bp were reduced to 27% and 16% respectively and the 202 bp product was not detected. None of the products were detected after 18 or 21 minutes.

Table 1: The effect of heating time on RR flour held at pH 3.3 determined using the multiplex PCR method

Time at 100°C and pH 3.3 (minutes)	Amount of PCR product[1]			
	80bp	**118bp**	**145bp**	**202bp**
0	100	100	100	100
3	74	77	73	67
6	57	58	21	6
9	36	23	24	15
12	67	33	47	21
15	48	27	16	0
18	0	0	0	0
21	0	0	0	0

[1] % product determined relative to amount at 0 minutes

A. Information taken from a CCFRA report[12] concerning the Roundup Ready Soya Multiplex PCR Assay

To eliminate any variation due to amount of DNA in each PCR reaction, the ratio of the lectin 80bp product to each of the other 3 products was determined for all experiments (Table 2) i.e. normalised with respect to the 80bp product. The ratios of each would be expected to remain constant if either no degradation of the target DNA occurred or if the degree of degradation between the 80bp target and the other targets was comparable. At pH 3.3 the ratios tended to increase with increasing heating time, suggesting that at low pH there were differences in the detectability of the three EPSPS targets compared to the smaller lectin target, with the 80bp target being degraded at a slower rate compared with the other targets. At pH 4.3, the 80/118bp and 80/145bp ratios decreased during the first 3-9 minutes of heating, then increased ,returning to the their original value, whereas the 80/202bp ratio increased with heating time. Similar trends were observed at pH 6.7 except for the 80/202bp ratio, where little change occurred. However, further analyses are required to replicate these observations and focus around the pH where an effect is observed. These initial results indicate that the different targets used in PCR are not detected equally in these experiments.

Other studies that have been carried out show similar results. Hupfer (1998) demonstrated that PCR detection of GM maize in polenta could be influenced by pH during thermal treatment of the product. They showed that detection of a 1,914 bp segment of the cry1A(b) gene was not possible after boiling at neutral pH for 30 minutes, whereas a 211bp fragment was detected after boiling for 105 minutes. At pH 2-3, the larger segment was not detected after boiling for 5 minutes and the smaller fragment was not detected after 15 minutes. At weakly alkaline conditions (pH 8.5-9.5) the larger segment was still detected after 60 minutes ,showing that the DNA was more resistant to hydrolysis under alkaline conditions. Straub *et a.l* (1999) looked at detection of DNA from GM starter organisms in thermally treated fermented sausages. Over a nine week storage period there was a decrease in the level amplification of a 1,322 bp target, whereas no decrease was detected in amplification of a 166bp target.

As a result of such observations, it is common practice to use small target sequences in screening methods for GMOs. Van den Eede *et al.* (2000) evaluated PCR methods for the detection of as little as 0.4% RR soya and Bt-176 maize in a range of processed foods. These included polenta (maize grit cooked at 100 ^{o}C for 45 minutes), infant formula (containing soya and maize powders heated at 100 ^{o}C for 45 minutes), biscuits (soya and maize cooked at 180 ^{o}C for 10 minutes) and acidified soybean meal (pH 4.5, heated at 60 ^{o}C for 10 minutes). PCR was performed using small target sequences

(118bp) found in the 35S promoter and NOS terminator. Fifty PCR cycles were needed to ensure detection of the target sequences within the products.

However, the work reported here suggests that there may be differences in amplification of small (<202bp) sequences under low pH conditions. Whilst this may not be that important for qualitative analysis, it is likely to have significance for the accuracy of quantitative analysis of processed foods with low levels of GM-DNA, when two target sequences are analysed simultaneously as in real-time PCR.

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Hupfer, C., Hotzel, H., Sachse, K., Engel, K-H. (1998) Detection of the genetic modification in heat-treated products of Bt maize by polymerase chain reaction. *Z. Lebensm Unters Forsch A 206:* 203-207.

Straub, J.A., Hertel, C., Hammes, W.P. (1999) The fate of recombinant DNA in thermally treated fermented sausages. *Eur Food Res Technol 210:*62-67.

Van den Eede, G., Lipp, M., Eyquem, F., and Anklam, E. (2000) Validation of an analytical method for the detection of GMO-derived DNA in processed foodstuffs. European Commission. EUR 19677 EN

A. Information taken from a CCFRA report[12] concerning the Roundup Ready Soya Multiplex PCR Assay

Table 2: The effect of heating time on RR flour held at pH 3.3, 4.3 and 6.7

determined using the multiplex PCR method

Time at 100°C and pH 3.3 (minutes)	Ratio lectin 80bp/RR-117bp	Ratio lectin 80bp/RR-150bp	Ratio lectin 80bp/RR-202bp
0	1.8	3	1.9
3	1.8	3	2.1
6	1.7	7.8	17.5
9	3	5	5
12	3.6	4.4	6
15	3.8	9	NP
18	NP	NP	NP

NP= no PCR products observed

Time at 100°C and pH 4.3 (minutes)	Ratio lectin 80bp/RR-117bp	Ratio lectin 80bp/RR-150bp	Ratio lectin 80bp/RR-202bp
0	2	4.4	1.9
3	2.2	2.9	1.8
6	1.3	2	1.9
9	1.3	2.2	2.3
12	1.5	2.6	2.6
15	1.8	3.7	2.7
18	1.9	3.9	3

Time at 100°C and pH 6.7 (minutes)	Ratio lectin 80bp/RR-117bp	Ratio lectin 80bp/RR-150bp	Ratio lectin 80bp/RR-202bp
0	1.8	4.2	1.7
3	1.7	3.9	1.6
6	1.2	2.3	1.5
9	1.5	2.4	1.7
12	1	1.9	1.3
15	1.2	2.1	1.3
18	1.4	2	1.4
21	1.6	2.4	1.4

Results represent mean values from 3 replicate PCR reactions using the RR multiplex assay.
Post-PCR analysis carried out using the Agilent 2100 Bioanalyser

Appendix B. DNA extraction data obtained using the Maxwell 16

Table 1. Comparison of DNA extraction data from the Maxwell 16 to data obtained with other methods

	Promega Maxwell 16 Robot		Standard Extraction	
Sample	**A260/A280nm Ratio**	**Conc. (ng/µl)**	**A260/A280nm Ratio**	**Conc. (ng/µl)**
Tortilla Chip	2.0	2	2.5 C	66
Processed Mixed Cereals	1.6	11	1.7 C	59
Dried Cake Mix Test material	3.0	6	1.9 W	327
Pastry test Material	1.7	5	1.8 C	250
Soya Flour	1.9	88	1.9 W	476
Animal Feed Test Material	1.8	44	1.8 C	245
TVP	1.8	54	1.8 W	239
Dry Mix	4.0	4	2.0 W	260

DNA extracted from ~50mg of sample and eluted into 400µl using the Promega Maxwell 16 Robot.
C = CTAB DNA extraction ~2g of sample and eluted into 40µl.
W = Wizard DNA extraction ~200mg of sample and eluted in to 50µl
Absorbance measurements were obtained with a spectrophotometer (Genequant Pro).
Good quality DNA should have a A260/A280 ration of between 1.8 and 2.0

Table 2. Replicate extracts performed on Certified Reference Materials

	Extraction by Maxwell 16 Robot	
Sample	**A260/A280nm Ratio**	**Conc. (ng/µl)**
0.1% RR Soya Standard (3 reps)	1.9	452
	1.9	454
	1.9	227
1% RR Soya Standard(3 reps)	1.9	749
	1.9	480
	2.0	385
5% RR Soya Standard(3 reps)	1.9	437
	1.9	358
	1.9	454

DNA extracted from ~50mg of certified RR soya standard and eluted into 400µl using the Promega Maxwell 16 Robot

Appendix B. DNA extraction data obtained using the Maxwell 16

Table 3. Replicate extracts performed on GeMMA round samples

	Extraction using Maxwell 16 Robot	
Sample	**A260/A280nm Ratio**	**Conc. (ng/µl)**
Soya flour	1.9	131
	1.9	116
	2.0	125
	2.0	124
Animal feed	2.3	16
	2.2	13
	1.9	15
	2.0	14
TVP	2.0	59
	2.0	51
	1.9	43
	1.9	84

DNA extracted from ~50mg of sample and eluted into 400µl using the Promega Maxwell 16 Robot.

Discussion

The Maxwell 16 is a magnetic bead based cartridge DNA extraction system that enables the extraction of up to 16 samples in approximately 30 minutes. It uses separate kits which contain the cartridges but the system requires no pre-set up, preparation of buffers or additional plastic. It is about the size of a microwave, making it suitable for a small laboratory.

The extraction is performed on approximately 50mg of homogenised sample, which is placed into the end well of the cartridge. The sample undergoes a sequence of lysis, extraction, purification and elution in the chambers of the cartridge. There is no hands-on time apart from placing the sample into the cartridge and pipetting out the elution buffer into a separate tube.

The Maxwell 16 produced DNA giving A260/A280 ratios of 1.8-2.3 from certified reference materials, soya flour, TVP and animal feed. The DNA concentration obtained was lower than with other standard methods, but this was probably due to the higher amount of eluent required to recover the DNA from the magnetic beads. The amount of DNA obtained from replicate extractions from these materials was very reproducible.

The yield of DNA extracted from samples like tortilla chips, dried bakery mixes , pastry and mixed cereals was <10 ng/µl and the A60/A280 of the dry bakery mix and cake mix were very high. This indicated that the DNA extraction was not optimal for these types of samples.

C. Presentation by Somerset Scientific Services covering use of the Roundup Ready Soya multiplex PCR assay

Semi quantification
of Roundup Ready Soya
by Multiplex PCR assay
using the Agilent 2100 Bioanalyser

Somerset Scientific Services
Yanina Pelegri Fairfax

The use of GMOs in foodstuffs has led to consumer demand for effective labelling and freedom of choice

Mandatory labelling of food and feed derived of GMO only allows an intentional contamination up to 0.9% for approved GMO or 0.5% for GMO which are not approved but have positive safety decision

The challenge for the Scientific community is to develop and validate analytical methods to detect and accurately quantify the presence of GMOs in a wide variety of raw and processed foods

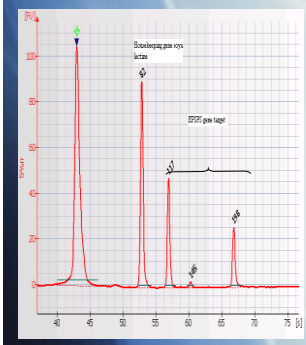
- PCR is the method of choice because it is rapid, sensitive and specific.
- Real-time PCR systems offer the greatest potential for accurate quantitative analysis, particularly as detection is prior to end point. (expensive)
- Quantification of PCR products by agarose gel electrophoresis coupled with densitometric analysis and documentation systems has been shown to be successful. However, there are still a great many limitations associated with quantitative PCR, particularly in relation to its accuracy and reproducibility.

We have observed a variation between Roundup Ready standards in the agarose gel but we were unable to quantify and produce a Standard curve.

We have been looking for alternatives and Agilent Technologies offer a new microfluidic capillary electrophoresis system, Agilent 2100 Bioanalyser, to assess their relative performance in the quantitative analysis of PCR products.

Presentation was given as part of a training course for Public Analysts on DNA methods and Lab-on-a-Chip CE held at Campden BRI on 19 & 20 April 2007.

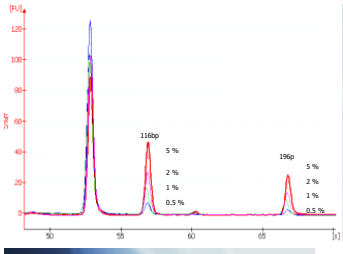
C. Presentation by Somerset Scientific Services covering use of the Roundup Ready Soya multiplex PCR assay



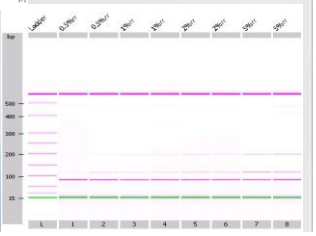
Multiple PCR reactions were performed for the semi-quantification of GM Soya:

• Single copy "housekeeping" sequence, soya lectin was used, as internal control to normalise the PCR in respect to the amount of total amplifiable soya DNA present.

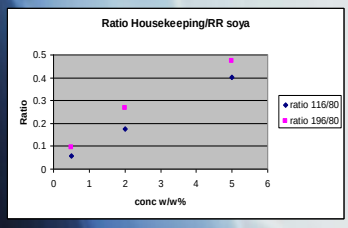
• GMO content was then determined by amplification of EPSPS(5-enolpyruvyl-shikamate-3-phosphate synthase) gene target of 117bp, 146bp, 198bp, only found in Roundup Ready GM soya from Monsanto.(**Steve Garrett " Analysis of genetically modified soya using the Agilent 2100 Bioanalyser**)



Certified reference material (CRMs) containing known amounts of Roundup Ready Soya were amplified to produce a Standard curve.



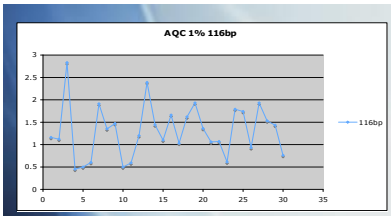
We estimated the levels of GM in an unknown material by comparing the ratio between lectin and the Roundup Ready targets in the sample and interpolating this in the calibration curve with the CRMs materials



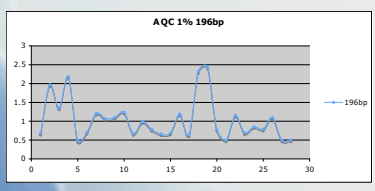
Sample	Ratio 116/80	Ratio 196/80
5% CRM	0.401	0.472
2% CRM	0.177	0.266
0.5% CRM	0.059	0.096

We check:

- reproducibility in the assays by analysing standards and samples in duplicate,
- between assays by running 1% Roundup Ready soya CRM as a AQC standard and extract it every time that we run a sample for semiquantification



	116bp	196bp
Mean	1.3	1.04
Std Desv	0.577	0.55
N	30	28



Our external control is GeMMA samples which has proven successful

sample	result	assigned value	z-score
GeM C09C	3.48	2.59	0.6
GeM S35-A	3.46	2.66	0.6
GeM C10	>2	5.34	
GeM C11	2.1	1.63	0.5
GeM S38-A	2.19	2.72	-0.5
GeM S39-A	>2	2.73	

Presentation was given as part of a training course for Public Analysts on DNA methods and Lab-on-a-Chip CE held at Campden BRI on 19 & 20 April 2007.

D. Results from the Public Analyst's trial of the Multiplex PCR GMO screening method

	Laboratory A Results		Laboratory B Results		Laboratory C Results		Laboratory D Results		Laboratory E Results		Laboratory F Results	
Sample	1/5	1/25	1/5	1/25	1/5	1/25	1/5	1/25	1/5	1/25	1/5	1/25
A.Maize flour(0.1% Bt11 maize)	80	80		80	81	81	81	83	79	79	81	80
	127/131				131	131	132	134			132	132
B. Spice mix - Possibly contains mustard (CaMV +ve)	80	79						83	79	79		
	105	104		106	108	107	108	110	103	102	107	107
	130	129		131	132	132	131	134	128	126	132	132
C .TVP (3%RR soya & trace amounts of maize)					82				80			
	89	88		90	91	94	91	92	88	85	90	90
	126/131	124/129		126/132	132	132	130	132	122/127	119/125	127/132	132
D. Maize flour (1% Bt 11 maize)	81	80		82	81	82	81	82	79	78	82	82
	126/131	124/129		126/132		132		132	122/129	120/126	127/132	133
E. Maize polenta (no GM)	81	80		81	82	82	82	82	80	78	81	82
									123/129			
F Animal Feed (3% RR soya & non GM maize)	81	80		81	83	84	82	92	80		81	82
	90	89		90	94	94		93	89	87	90	91
	127/132	125/130		125/131	134	134	134	132	123/129	120	132	128/132
GM soya/GM maize/CAMV DNA control		80		80		82		83		80		81
		88		88		93		93		89		90
		104		105		111		110		105		107
		130		124/130		133		134		123/129		132
0.5%GM soya reference flour									80			
	90	88		89	94	93	92	93	88	86		89
	127/131	123/130		124/130	133	133	133	133	123/129	120		128

see next page for explanation of values found in the table

Analysis was performed by nine UK Public Analysts laboratories and the Laboratory of the Government Chemist during July-September 2008

D. Results from the Public Analyst's trial of the Multiplex PCR GMO screening method

	Laboratory G Results		Laboratory H Results		Laboratory I Results		Laboratory J Results	
sample	1/5	1/25	1/5	1/25	1/5	1/25	50ng/µl[1]	10ng/ µl[1]
A.Maize flour(0.1% Bt11 maize)	82	81	79	80	81	82	*Detected*[2]	82
	133	132	124/130	130	131	127	*Detected*[2]	132
B. Flavour mix (CaMV +ve)	83	83						
	88							
	111	112	105	104	106	107	110	133
	132	133	130	132	131	132	111	13
C .TVP (3%RR soya & trace amounts of maize)	83	82	80		81			
	95	93	89	90	90	89	94	93
	134	133	123/129	121/128	127/131	127/131	134	131
D. Maize flour (1% Bt 11 maize)	84	83	80	80	81	81	83	84
	135	133	123/129	121/128	131	126/131		134
E. Maize polenta (no GM)	85	83	79	80	81	81	82	83
F Animal Feed (3% RR soya & non GM maize)	85	83	80	80[3]	81	81	83	84[3]
	96	94	89	88	91	90	93	93
	114							
	136	134	123/129	121/127	128/132	126/131	133	130
RR soya/GM maize/CAMV DNA control		83		80		82		81
		93		89		90		91
		109		105		107		109
		133		125/131		127/132		132
0.5%RR soya reference flour								
	95	94	90	91	90	90	92	92
	134	135	126/130	125/131	127/132	127/132	132	132

PCR product results (size in bp) are given for 1/5 and 1/25 dilutions of the sample DNA extracts following amplification with the Multiplex PCR GMO screening assay

PCR products are reported in the following order by each laboratory-

Target sequence	Theoretical size (bp)	Series II DNA 1000 labchip Size (bp)*
zein	68	80
lectin	80	88
CaMV	104	104
Nos terminator 35S promoter	118 123	125 130 (can appear as one peak)

* based on Campden BRI data

The yellow filled boxes are used to indicate the absence of an anticipated PCR product.

The blue filled boxes are used to indicate the presence of additional PCR products to those anticipated.

The grey filled boxes indicate that no results were submitted.

[1] Laboratory quantified DNA extracts. [2] Laboratory reported inconclusive results but indicated they had detected the target. [3]Detected in 1/3 replicates only.

Analysis was performed by nine UK Public Analysts laboratories and the Laboratory of the Government Chemist during July-September 2008

FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE FOR THE DETECTION OF ROUNDUP READY SOYA USING A MULTIPLEX PCR ASSAY

Prepared by

Campden BRI

Appendix E: Standard Operating Procedure for the detection of Roundup Ready Soya using the Campden BRI multiplex PCR Assay

CONTENTS

Appendix E: Standard Operating Procedure for the detection of Roundup Ready Soya using the Campden BRI multiplex PCR Assay

1. HISTORY / BACKGROUND

Genetically Modified Organisms (GMOs) contain DNA sequences which are distinct from those in the naturally occurring organism. They carry a synthetic DNA construct made up of a gene conferring a novel trait and gene regulatory elements, promoters and terminators which switch on and off expression of the gene. Some GMO's also contain marker genes such as antibiotic resistance genes, introduced into the organism to enable selection of the novel trait.

These novel sequences can be used to detect the GMO. The polymerase chain reaction can be used to amplify these sequences, enabling detection of the organism's DNA even when present at low levels. Presence of these sequences indicates that the sample contains material derived from a GMO.

2. PURPOSE

This method allows the identification of Roundup Ready soya in soya based food and ingredients. It can be used in a semi-quantitative manner, to establish whether the level of Roundup Ready soya is present at trace, low or high levels. It is only recommended for use by laboratories who do not have the ability to perform Roundup Ready soya quantification using real-time PCR.

3. SCOPE

The method allows the semi-quantitative estimation of the level of Roundup Ready soya in flours and some processed foods and ingredients. Sample types include mixed flours, TVP, animal feed and baked products.

4. DEFINITIONS AND ABBREVIATIONS

DNA : Deoxy-ribonucleic acid. This molecule comprises strings of the four bases (G, A, T, C) forming genes. Referred to as the blue-print of life.

dNTP : deoxy-nucleotide triphosphates. An abbreviation for any of the four bases forming DNA.

PCR : Polymerase Chain Reaction – a method of amplifying a single DNA fragment to produce millions of copies, which can be detected.

Primer : A short oligonucleotide designed to anneal to specific regions of DNA in order to facilitate the PCR. Primers are designed to complement regions of DNA bounding the gene of interest.

SDW : Sterile distilled water of molecular biology grade.

***Taq* polymerase :** A specific, heat-stable DNA polymerase used to replicate DNA targets during PCR

5. PRINCIPLE OF THE METHOD

The polymerase chain reaction (PCR) is used to detect DNA sequences in living organisms and in materials derived from living organisms. It relies on the binding of single-stranded DNA primers to a specific DNA target sequences and the copying of this target in the presence of excess amounts of DNA subunits (nucleotides) and a DNA polymerase (Taq). Multiple cycles at specific temperatures result in the million-fold copying of the target sequence. Size separation and detection of the amplification products is performed using lab-on-a-chip capillary electrophoresis using (Agilent 2100 Bioanalyzer). The size and concentration of the amplification products are then compared to amplification products produced from certified reference materials.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

All reagents should be of a suitable purity defined for molecular biology analysis (e.g. Sigma molecular biology products).

It is recommended that a hot start Taq polymerase, such as AmpliTaq Gold (Applied Biosystems, Warrington, UK) is used for PCR.

Reagents for PCR are stored in a dedicated PCR reagent freezer at –15 ^{o}C to-22 ^{o}C for up to six months, unless otherwise stated.

The water used should be sterile molecular biology grade. 2ml aliquots of Sigma molecular grade water are stored frozen for up to 6 months

6.2 Solutions, standards and reference materials

Solutions 6.2.2–6.2.7 should be prepared in a laminar flow cabinet located in the PCR set up area. The cabinet should be decontaminated using UV irradiation. Latex gloves should be worn throughout the procedure.

6.2.1 4mM dNTP mixture

dCTP, dGTP, dATP, dTT, bought as individual 100mM solutions.

Appendix E: Standard Operating Procedure for the detection of Roundup Ready Soya using the Campden BRI multiplex PCR Assay

Using a suitable pipette, add 38µl of each 100mM stock dNTP to a sterile labelled 1.5ml Eppendorf. Use suitable pipettes to add 800µl of water and aspirate gently to mix.

Use a suitable pipette to aliquot into labelled portions of 200µl in sterile 0.5ml Eppendorf tubes.

Store at -15°C to -22°C for up to six months.

6.2.2 20µM Primer solutions

TABLE 1 : Primer specifications

Primer set	Primer code	Size (bp)
RR202	**RR-202-For** 5'- CCG CAA ATC CTC TGG CCT T -3' **RR-202-Rev** 5'- GAT GAT CCA GGT GTC GCC TT -3'	200bp
RR150	**RR-150-For** 5'- ATG ATC GAC GAA TAT CCG ATT CTC -3' **RR-150-Rev** 5'- ATC GCA ATC CAC GCC ATT -3'	150bp
RR117	**RR-117-For** 5'- GGT CTA CGA TTT CGA CAG CAC CTT -3' **RR-117-Rev** 5'- TCA CCG TCT TCC GAT TTC ACC T -3'	117bp
LECTIN	**SLTM-1** 5'- AAC CGG TAG CGT TGC CAG -3' **SLTM-2** 5'- AGC CCA TCT GCA AGC CTT T -3'	80bp

Dilute the primers specified in Table 1, using water according to the manufacturer's instructions, to produce a primer concentration of 100µM, thoroughly agitate until dissolved. The solution may be stored at this point at -15°C to -22°C for up to 1 year.

Using a suitable pipette, dilute 10µl of the primer solution in a labelled sterile 0.5ml Eppendorf using 90µl of water to give a working primer solution of 10µM. Dilute further as necessary.

Both the primer solution at 100µM and the working primer solution at 10µM can be stored in a dedicated PCR freezer at -15°C to -22°C for up to 6 months.

6.2.3 Ampli-Taq PCR Buffer

100mM Tris-HCl, 500mM KCl (as purchased-10x concentrate)
Store in at -15°C to -22°C for up to six months.

6.2.4 25 mM Magnesium Chloride ($MgCl_2$)

Store in a dedicated PCR freezer at -15°C to -22°C for up to six months.

6.2.5 5 Unit/µl Ampli-Taq Gold DNA polymerase

Store in a dedicated PCR freezer at -15°C to -22°C for up to six months.

6.2.6 PCR Mastermix

A PCR mastermix is prepared for the analysis of a batch of several samples. Remove aliquots of each reagent from the freezer and allow to thaw in the laminar flow cabinet.

Prepare the mastermix using the reagents and volumes detailed in the table below. Add the reagents to a sterile 2ml Eppendorf tube and mix thoroughly by gentle pipette aspiration prior to use.

The mastermix solution can be used immediately or stored (without addition of Taq Gold) in labelled aliquots and kept at -15ºC to -20ºC for up to 3 months. Aliquots must be thawed completely before use.

TABLE 2 : Example of preparation of PCR Mastermix

Reagents	Initial Concentration	Final Concentration in PCR reaction (25µl reaction vol.)	Equivalent in a single reaction(µl)
PCR Buffer	10x	1x	2.5
$MgCl_2$	25mM	3mM	3.0
dNTPs	4mM	125 µM	1.0
Each Primer	10µM	300nM	0.75
Water	-	-	7.25
Volume			**19.75**
TaqGold	5U/µl	0.05U/µl	0.25
DNA			5.0
Total			25.00

6.2.7 80%(v/v) Ethanol

Using a 100ml measuring cylinder measure 80ml of ethanol and place in a labelled Schott bottle.
Using a 100ml measuring cylinder measure 20ml water and add to the Schott bottle, and mix by gentle shaking.
This can be stored at room temperature from 1 year.

6.2.8 Haz Tabs Working Solution.

Dissolve 2 tablets of Haz Tab in a 1 litre of tap water.
This can be store in a Schott bottle for up to 1 year.

6.3 Commercial kits

6.3.1 Agilent DNA 1000 Kit.

Available from Agilent Technologies, catalogue number 5067-1504
Kit Contains: 1vial DNA ladder
2 vials DNA marker
1 vial Dye concentrate
3 vials DNA gel Matrix.

6.3.2 Agilent DNA Chips

Available form Agilent Technologies, catalogue number 5067-1505
Contains: 25 chips
1 electrode cleaning chip.

6.4 Plasticware

All equipment required to be autoclaved is sterilised using the following conditions: 121°C±2.5°C for 15 min±2min at 1.0 Bar.

The following items are sterilised by autoclaving

2ml Eppendorf tubes

1.5 ml Eppendorf tubes

0.5 ml Eppendorf tubes

The following items are UV sterilised for 5 minutes using the UV light source in a laminar flow cabinet;

PCR tube and caps strips (eight reactions each)

PCR tube storage block for twenty four tubes

6.5 Equipment

Thermocycler

Laminar flow hood

Sets of precision pipettes (including P10, P20, P100, P200, P1000)

Benchtop whirlimixer

Sterile filter pipette tips

Micro-centrifuge

Agilent 2100 Bioanalyser

Vortex Mixer-IKA model MS2-S8/S9

7. PROCEDURES

7.1.1 Preparation of sample DNA

Samples and Roundup Ready soya reference materials are extracted using a method suitable for the extraction of DNA from foods. Such methods are found in the British Standard for DNA extraction from genetically modified organisms (BS EN ISO 21571:2005. Available from www.bsi-global.com). Commercial kits by Tepnel Biosystems, Promega, Qiagen and Genescan are also suitable for this purpose. Additionally, the Promega Maxwell^{TM} 16 Robot can be used for some food matrices.

DNA should be diluted to give a final concentration of 10ng/µl for soya flour samples.

It is suggested that Roundup Ready soya 0.1, 1.0 and 5% w/w reference materials are extracted along with the samples. A positive control (relating to the samples) and extraction negative control should also be analysed.

7.1.2 Amplification of target sequence

In a laminar flow hood

(Use sterile filter tip pipette tips and wear disposable gloves during the procedure.)

Remove all used tips and tubes; tube holders and wipe laminar flow hood with sterilising solution (Haz Tabs and 80% Ethanol). Replace tips, tubes and tube holder.

UV sterilise all equipment and empty tubes in the PCR laminar flow cabinet.

Make up PCR mastermix as outlined in Mastermix preparation, (allowing 10% for pipetting errors) and mix thoroughly by vortexing.

Using a suitable pipette, add 20µl aliquots of PCR mastermix to PCR tube strips contained in a UV sterilised PCR tube set-up block. Using a suitable pipette, add 5µl of each diluted DNA to PCR tubes according to the plate layout.

Fit PCR strip caps to tube strips and secure using capping tool if necessary.

Remove all used tips and tubes; tube holders and wipe laminar flow hood with sterilising solution (Haz Tabs and 80% Ethanol). Replace tips, tubes and tube holder.

Appendix E: Standard Operating Procedure for the detection of Roundup Ready Soya using the Campden BRI multiplex PCR Assay

Transfer PCR tubes to the thermocycler laboratory.

In thermocycler laboratory

Set the PCR programme (Table 3) and leave until the program has finished (approximately 3 hours or overnight).

After the PCR programme is complete, remove tubes from thermocycler and store samples at 1ºC to 6ºC for up to 2 days. Alternatively, PCR products can be stored for up to three months at between –15ºC and –22ºC.

Note: Do not remove PCR products from the thermocycler laboratory.

Table 3

	Temperature	**Time**
RR Multiplex Assay	95°C	10min
	94°C 60ºC 72ºC	30 sec 30 sec **40 cycles** 25sec
	72ºC	7 min
	4ºC	HOLD

PCR products are detected using the Agilent Bioanalyser 2100.

7.2 Detection of PCR product using the Agilent 2100 Bioanalyzer

For complete instructions on using the Agilent 2100 Bioanalyser, see manufactures instructions.

Remove the ladder, markers and gel matrix from the DNA1000 LabChip reagent Kit and leave to warm to room temperature for 30 minutes.

Prime DNA1000 LabChip according to manufacturer's instructions using prepared gel matrix.

Use a suitable to load 5µl of size markers into all sample wells and well labelled with a ladder symbol, ensuring marker settles onto bottom of well and does not remain on sides.

Using a suitable pipette, load 1µl of ladder into the well labelled with a ladder symbol, ensuring ladder settles onto bottom of well and does not remain on sides.

Using a suitable pipette, load 1µl of PCR product into one of the 12 sample wells, 1 – 12. Ensure samples have settled onto bottom of well and have not remained on sides of well. Fill any spare wells with 1µl of size marker.

Appendix E: Standard Operating Procedure for the detection of Roundup Ready Soya using the Campden BRI multiplex PCR Assay

The PCR products from the 3 Roundup Ready Soya reference materials (0.1, 1.0 & 5%) must also be run on each chip containing samples.

Use the IKA vortex mixer to vortex the chip for 1 minute at 2,400 rpm, then load into slot in 2100 Bioanalyser.

Select chip assay type as DNA1000 assay. Press start when chip is ready and wait for 1-2 minutes to ensure analyser starts and there are no problems with chip.

If chip error is reported:

a. Stop run and remove chip.

b. Check chip wells to ensure samples are in bottom of wells and are not adhering to sides. If sample is on sides use a pipette to move it into base of well. Reload chip into analyser and restart run.

c. If all samples are in bottom of wells invert chip and examine chip wells for bubbles. If chip contains bubbles discard chip and reload samples into fresh chip.

d. If problems persist and there are no obvious problems consult your line manager. you may need to run a full instrument diagnostics test.

After run is complete save file into appropriate folder. Remove DNA chip from analyser and clean analyser pins with cleaning chip containing approximately 350µl SDW.

Print the Bioanalyzer report for the analysis, including gel-like image, PCR product fragment sizes and concentrations. Fix in laboratory notebook.

Repeat for additional chips required to run all samples.

8. DATA ANALYSIS

Review the data from each chip and compare fragment sizes of the samples to the size of the Roundup Ready amplification products obtained with the reference material

Primer set	**Theoretical size**	**Observed size (bp)**
RR202	200	206
RR150	150	149
RR117	117	122
LECTIN	80	88

Sizes of the fragments may vary slightly between different chips run but should be within a tolerance of ±5%.

In samples containing no Roundup Ready soya only the lectin fragment should be detected. In samples with trace levels of Roundup Ready Soya the 80bp lectin fragment should be detected and the 117bp fragment should be just visible but not necessarily detected by the Bioanalyser.. In samples where the 117, 150 and 202bp fragments are detected the levels of Roundup Ready soya are higher. A figure showing amplification products produced by reference materials is found Appendix 11 A.

It is also possible to estimate levels of Roundup Ready Soya in a sample by comparing the ratio of the 117bp product to the 80bp lectin product to ratios produced from reference materials. In order to do this, take the concentration of the 117bp product (found on the Bioanalyzer report) and divide by the concentration of the of 80bp product. Determine this ratio for each sample and reference material. Plot the ratio against the % Roundup Ready soy to produce a calibration curve and determine the values for the samples. A typical calibration curve is shown in Appendix 11B.

Samples can be reported as containing either <0.1%, between 0.1 and 1.0%, between 1.0 and 5%, or >5% Roundup Ready soya.

9. QUALITY CONTROL

9.1 PCR Negative Controls

A PCR negative must be used as a method control with every set of samples amplified at the same time. For a PCR negative, 5µl sterile water replaces the sample DNA extract, when setting up the PCR.

The purpose of the PCR negative is to identify if contamination has occurred during the PCR procedure. The PCR negative is usually manipulated last at each stage of the process, to pick up any possible source of contamination.

9.2 Extraction Negative Control

An extraction negative showing any PCR products similar to those anticipated for Roundup Ready soya means the PCR batch is invalid and all samples must be re-amplified.

Both the extraction negative and PCR negative should show no PCR product present. Presence of the PCR product indicates contamination has occurred and the PCR batch is invalid and all samples must be re-amplified.

9.3 PCR Positive Controls

PCR positive control (i.e. DNA that has been extracted from material of a known Roundup Ready Soya content) must be run with each batch.

If the positive control sample contains a high level of Roundup Ready soya (5% (w/w)) four PCR products corresponding to 70, 117, 150 and 200bp should be observed.

9.4 Primer Quality Control

New primers should be individually be tested on a wide range of reference materials either before or during sample analysis.

9.5 2100 Bioanalyzer – Quality Control

A ladder with thirteen DNA fragments ranging in size from 15bp to 1500bp should be fully resolved and detected by the analyser using default settings

10. PRECISION AND ACCURACY

All sample DNA extracts are amplified in duplicate and the results should be consistent with each other. For Roundup Ready soya reference materials, the amount of the 117bp PCR product and the 117/80 ratio should increase with increasing levels of Roundup Ready soya. The Agilent Bioanalyzer has a stated tolerance of 30% for accuracy of PCR product quantification. As the 117/80 ratio is based on the concentration of two products, a combined variation of up to 60% would be expected for the determined level of Roundup Ready soya in a control sample.

11. APPENDICES

A. Application of the Roundup Ready Soya Multiplex PCR assay to certified reference materials

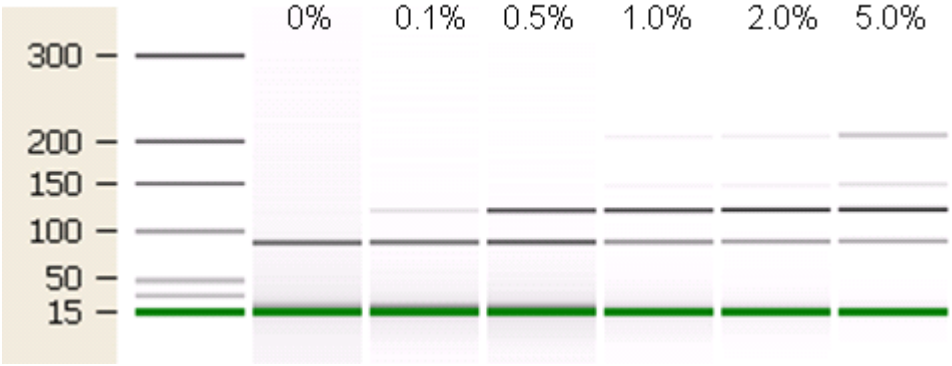
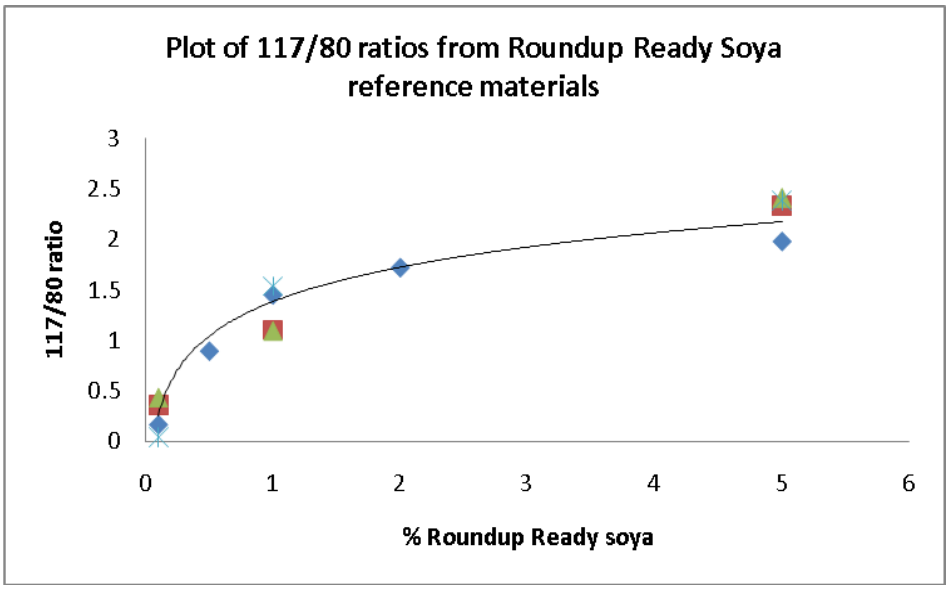


Figure shows a gel-like image generated using the Bioanalyzer. It shows PCR products from certified reference materials (0-5%(w/w)) and a DNA ladder (bp). There is an increase in the amount of the 117, 150 & 202bp products with increasing levels of Roundup Ready soya.

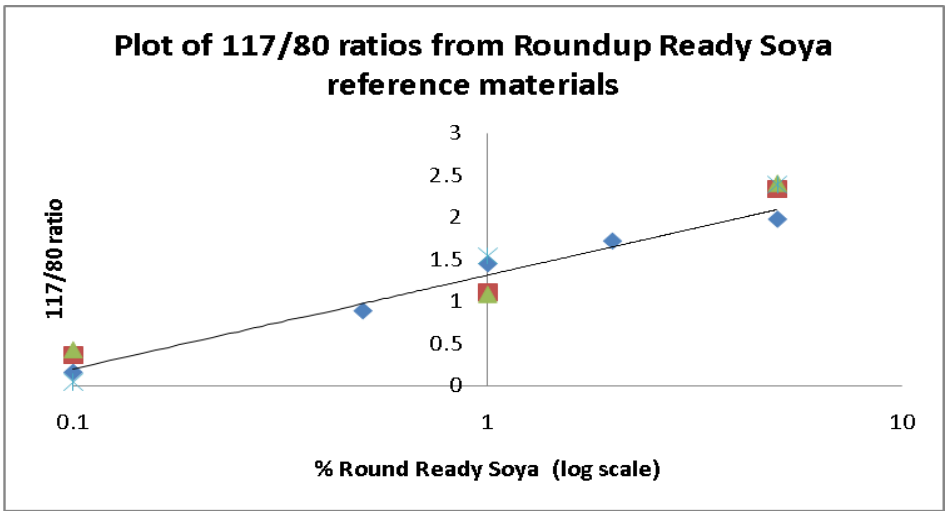
Appendix E: Standard Operating Procedure for the detection of Roundup Ready Soya using the Campden BRI multiplex PCR Assay

B Calibration curves produced by the Roundup Ready Soya Multiplex PCR assay

1



2



These graphs show 117/80bp ratio data produced from the analysis of 0.1%, 1.0% & 5% Roundup Ready certified reference materials analysed on four separate occasions. The 0.5% and 2% references have been analysed on one occasion.

1. Shows the 117/80 ratios reaching a plateau with higher levels of Roundup Ready Soya

2. A linear relationship can be achieved using a log scale on the % Roundup Ready Soya axis.

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FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE FOR THE SIMULTANEOUS DETECTION OF GENERAL DNA TARGETS ASSOCIATED WITH THE PRESENCE OF GENETICALLY MODIFIED SOYA AND MAIZE

Prepared by

Campden BRI

Appendix F: Standard Operating Procedure for the simultaneous detection of general DNA targets associated with the presence of genetically modified soya and maize

CONTENTS

Appendix F: Standard Operating Procedure for the simultaneous detection of general DNA targets associated with the presence of genetically modified soya and maize

1. HISTORY / BACKGROUND

Genetically Modified Organisms (GMOs) contain DNA sequences which are distinct from those in the naturally occurring organisms. They carry a synthetic DNA construct made up of a gene conferring a novel trait and gene regulatory elements; promoters and terminators which switch on and off gene expression.

The polymerase chain reaction (PCR) can be used to amplify these sequences, enabling detection of the organism's DNA even when present at low levels. Presence of these sequences indicates that the sample either contains DNA derived from a GMO or it contains DNA from a naturally occurring organism that has the regulatory sequences.

2. PURPOSE

A simple, cost effective, GMO screening method enabling the simultaneous detection of DNA targets associated with the presence of GM soya and maize. It also allows detection of CaMV, a potential cause of false positives when carrying out the CaMV 35S promoter screen. It cannot be used for GMO quantification purposes.

3. SCOPE

The method allows detection of specific GM DNA markers (CaMV35S promoter and Nos terminator) and endogenous markers (lectin and zein) found in raw materials derived from GM soya and maize. It is also suitable for screening some processed foods, animal feeds and ingredients.

4. DEFINITIONS AND ABBREVIATIONS

CaMV: Cauliflower Mosaic Virus

DNA : Deoxy-ribonucleic acid. This molecule comprises strings of the four bases (G, A, T, C) forming genes. Referred to as the blue-print of life.

dNTP : deoxy-nucleotide triphosphates. An abbreviation for any of the four bases forming DNA.

PCR : Polymerase Chain Reaction – a method of amplifying a single DNA fragment to produce millions of copies, which can be detected.

Primer : A short oligonucleotide designed to anneal to specific regions of DNA in order to facilitate the PCR. Primers are designed to complement regions of DNA bounding the gene of interest.

SDW : Sterile distilled water of molecular biology grade.

***Taq* polymerase :** A specific, heat-stable DNA polymerase used to replicate DNA targets during PCR

5. PRINCIPLE OF THE METHOD

The polymerase chain reaction (PCR) is used to detect DNA sequences in living organisms and in materials derived from living organisms. It relies on the binding of single-stranded DNA primers to a specific DNA target sequence and the copying of this target in the presence of excess amounts of DNA subunits (nucleotides) and a DNA polymerase (Taq). Multiple cycles at specific temperatures result in the million-fold copying of the target sequence. Size separation and detection of the amplification products is performed using lab-on-a-chip capillary electrophoresis using (Agilent 2100 Bioanalyzer). The size of the amplification products are then compared to sizes of amplification products produced from reference materials.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

All reagents should be of a suitable purity defined for molecular biology analysis (e.g. Sigma molecular biology products). The water used should be sterile molecular biology grade.

6.2 Solutions, standards and reference materials

Solutions should be prepared in a laminar flow cabinet. The cabinet should be decontaminated using UV irradiation. Latex gloves should be worn throughout the procedure.

6.2.1 QIAGEN® Multiplex PCR kit

2x QIAGEN Multiplex PCR Mastermix, containing:

Hot StarTaq® DNA Polymerase

Multiplex PCR Buffer(containing 6 mM $MgCL_2$)

Store at -15°C to -22°C for up to 12 months.

Catalogue no. 206143

6.2.2 Primer Mastermix Solution

Obtain primers with the sequences found in Table 1.

TABLE 1 : Primer specifications

CaMV 35S promoter	35S-CF3 for	CCA CGT CTT CAA AGC AAG TGG
	35S-CR4 rev	TCC TCT CCA AAT GAA ATG AAC TTC C
Nos terminator	HA-NOS 118 for	GCA TGA CGT TAT TTA TGA GAT GGG
	HA-NOS 118 rev	GAC ACC GCG CGC GAT AAT TTA TCC
Soya lectin gene	STLM-1	AAC CGG TAG CGT TGC CAG
	STLM-2	AGC CCA TCT GCA AGC CTT T
Maize zein gene	ZET-M1	TGT TAG GCG TCA TCA TCT GTG
	ZET-M2	TGC AGC AAC TGT TGG CCT TAC
CaMV	CaMV1156F	AAG CAA AGA CCC TTC GGA GT
	CaMV1659R	CCT TTA GTT GGC TCG AGT AAT CA

Dilute the primers specified in Table 1 (10 in total), using sterile water according to the manufacturer's instructions, to produce a primer concentration of 100µM, thoroughly agitate until dissolved. The solution may be stored at this point at -15°C to -22°C for up to 1 year.

Appendix F: Standard Operating Procedure for the simultaneous detection of general DNA targets associated with the presence of genetically modified soya and maize

Using a suitable pipette, dilute 20µl of each 100µM primer solution in a labelled sterile 0.5ml Eppendorf using 80µl of sterile water to give a working primer solution of 20µM.

Using a suitable pipette, take 5 µl of each 20 µM primer solution and add to a single 0.5ml tube. Then add 50 µl of sterile water to give a total volume of 100 µl. This solution is 1 µM with respect to each primer and is known as the primer mastermix.

All the primer solutions at 100µM, 20 µM and the primer mastermix solution can be stored in a dedicated PCR freezer at -15°C to -22°C for up to 6 months.

6.2.3 PCR Mastermix

A PCR mastermix is prepared for the analysis of a batch of several samples.

Remove aliquots of each reagent from the freezer and allow to thaw in the laminar flow cabinet.

Prepare the mastermix using the reagents and volumes detailed in the table below and scale up according to the number of samples, positive and negative controls. Add the reagents to a sterile 1.5ml tube and mix thoroughly by gentle pipette aspiration prior to use.

TABLE 2 : Example of preparation of PCR Mastermix

Reagents	Initial Concentration	Final Concentration in PCR reaction (15µl reaction vol.)	Equivalent in a single reaction(µl)
QIAGEN multiplex PCR Mastermix	2x	1x	7.5
Primer mastermix	1µM for each primer	100nM	1.5
Sterile water	-	-	4
Volume			**13**
DNA			2.0
Total			15.00

Note the total volume recommended for the PCR reaction is 15 µl (including the DNA). This volume can be reduced or increased if required.

6.3 Commercial kits

6.3.1 Agilent DNA 1000 Kit.

Available from Agilent Technologies, catalogue number 5067-1504
Kit Contains: 1vial DNA ladder
2 vials DNA marker
1 vial Dye concentrate
3 vials DNA gel Matrix.

6.3.2 Agilent DNA Chips

Available form Agilent Technologies, catalogue number 5067-1505

Contains: 25 chips
1 electrode cleaning chip.

6.4 Plastic ware

All equipment required to be autoclaved is sterilised using the following conditions: 121°C±2.5°C for 15 min±2min at 1.0 Bar.

The following items are sterilised by autoclaving

2ml Eppendorf tubes

1.5 ml Eppendorf tubes

0.5 ml Eppendorf tubes

The following items are UV sterilised for 5 minutes using a UV light source in a laminar flow cabinet;

PCR tube and caps strips (eight reactions each)

PCR tube storage block for twenty four tubes

6.5 Equipment

Thermocycler

Laminar flow hood

Sets of precision pipettes (including P10, P20, P100, P200, P1000)

Bench top whirl mixer

Sterile filter pipette tips

Micro-centrifuge

Agilent 2100 Bioanalyser

Vortex Mixer-IKA model MS2-S8/S9

7. PROCEDURES

7.1.1 Preparation of sample DNA

Samples are extracted using an appropriate method for the extraction of DNA from food. Standard CTAB extraction, kits by Promega, Tepnel, R-Biopharm or QIAGEN would be suitable.

Dilute the extracted DNA to 1/5 (10 µl DNA extract plus 40 µl sterile water) and to 1/25 (10 µl of 1/5 dilution plus 40 µl sterile water).

Alternatively if you can quantify the DNA, dilute the DNA to about 50ng/µl and 10ng/µl.

7.1.2 Amplification of target sequence

UV sterilise all equipment and empty tubes in the PCR laminar flow cabinet.

Make up PCR mastermix as outlined in Mastermix preparation

Using a suitable pipette, add 13µl aliquots of PCR mastermix to PCR tube strips contained in a UV sterilised PCR tube set-up block. Using a suitable pipette, add 2µl of each diluted DNA (1/5 and 1/25 dilutions) to PCR . Use 2 µl of sterile water for PCR negative control.

Fit PCR strip caps to tube strips and secure using capping tool if necessary.

Transfer PCR tubes to the thermocycler laboratory.

In thermocycler laboratory

Set the PCR programme and leave until the program has finished (approximately 3 hours or overnight).

	Temperature	**Time**
GMO Multiplex Assay	95ºC	15 min
	95°C 62ºC 72ºC	25 sec 30 sec **40 cycles** 45sec
	72ºC	7 min
	4ºC	HOLD

After the PCR programme is complete, remove tubes from thermocycler and store samples at 1ºC to 6ºC for up to 2 days. Alternatively, PCR products can be stored for up to three months at between –15ºC and –22ºC.

Note: Do not remove PCR products from the thermocycler laboratory.

7.2 Detection of PCR product using the Agilent 2100 Bioanalyser

For complete instructions on using the Agilent 2100 Bioanalyser, see manufacturer's instructions.

Remove the ladder, markers and gel matrix from the DNA1000 LabChip reagent Kit and leave to warm to room temperature for 30 minutes.

Prime DNA1000 LabChip according to manufacturer's instructions using prepared gel matrix.

Use a suitable pipette to load 5µl of size markers into all sample wells and well labelled with a ladder symbol, ensuring marker settles onto bottom of well and does not remain on sides.

Using a suitable pipette, load 1µl of ladder into the well labelled with a ladder symbol, ensuring ladder settles onto bottom of well and does not remain on sides.

Using a suitable pipette, load 1µl of PCR product into one of the 12 sample wells, 1 – 12. Ensure samples have settled onto bottom of well and have not remained on sides of well. Fill any spare wells with 1µl of size marker.

The PCR products from the reference DNA (containing GM soya, GM maize and CaMV) must also be run on each chip containing samples.

Use the IKA vortex mixer to vortex the chip for 1 minute at 2,400 rpm, then load into slot in 2100 Bioanalyser.

Select chip assay type as DNA1000 assay. Press start when chip is ready and wait for 1-2 minutes to ensure analyser starts and there are no problems with chip.

If chip error is reported:

a. Stop run and remove chip.

b. Check chip wells to ensure samples are in bottom of wells and are not adhering to sides. If sample is on sides use a pipette to move it into base of well. Reload chip into analyser and restart run.

c. If all samples are in bottom of wells invert chip and examine chip wells for bubbles. If chip contains bubbles discard chip and reload samples into fresh chip.

d. If problems persist and there are no obvious problems you may need to run a full instrument diagnostics test.

After run is complete save file into appropriate folder. Remove DNA chip from analyser and clean analyser pins with cleaning chip containing approximately 350µl sterile water.

Print results of analysis, including gel image and report of fragment sizes.

Repeat for additional chips required to run all samples.

8. DATA ANALYSIS

Review the data from each chip and compare fragment sizes of the samples to the size of the amplification products obtained with the PCR positive control.

Primer set	**Theoretical size**	**Observed size (bp) on Series II DNA 1000 labchip**
CaMV	104	104
CaMV 35S promoter	123	130 (sometimes appears as a double peak with Nos terminator)
NOS terminator	118	125(sometimes appears as double peak with 35S promoter)
lectin	80	88
zein	68	80

NOTE-THERE IS ALSO A PRIMER DIMER PEAK WHICH RUNS AT ABOUT 50bp. IT MAY NOT ALWAYS BE OBSERVED IF HIGH LEVELS OF TARGET DNA IS PRESENT.

Sizes of the fragments may vary slightly between different chips runs but should be within a tolerance of ±5%

9. QUALITY CONTROL

9.1 PCR Negative Controls

A PCR negative must be amplified at the same time as a batch of samples. For a PCR negative, 2µl sterile Milli-Q water replaces the sample DNA extract, when setting up the PCR. It should not give any amplification products apart from primer dimers at about 50bp.

9.2 Extraction Negative Control

An extraction negative (no sample added) should be extracted with each batch of samples. It should not give any amplification products apart from primer dimers at about 50bp.

9.3 PCR Positive Control

PCR positive control containing a mixture of DNA from Roundup Ready soya, GM maize and CaMV. The DNA should produce PCR amplification products for the CaMV 35S promoter, Nos terminator, CaMV, lectin and zein targets.

9.4 2100 Bioanalyser – Quality Control

A ladder with thirteen DNA fragments ranging in size from 15bp to 1500bp should be fully resolved and detected by the analyser using default settings

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