

Final Report

Plasmid Standards for Real Time PCR and GM Enforcement Testing

T. R. Allnutt, J. Chisholm, H. Hird, S. Oehlschlager & C. M. Henry.

Executive Summary

This report details the progress in obtaining and assessing plasmids for use as real-time PCR standards and positive PCR controls. The project was originally conceived to validate and investigate the accreditation of European Network of GMO Laboratories (ENGL) plasmids. However, due to their unavailability in the time-scale of the project we have developed in-house methods to produce a selection of plasmids which will enable their use to be assessed across the major EU GM crop species, with particular emphasis on oilseed rape. The methodologies developed have proved successful and will enable our GM testing laboratories to produce plasmid reference materials for new GM events in a short space of time compared to the current production of Certified Reference Materials (CRMs). Eight plasmids have been made and standard PCR and real-time PCR analyses completed. These plasmids include one which enables positive control PCRs for identification of cauliflower mosaic virus contamination (which can confound p35s tests); three endogenous plant gene quantification plasmids; and four GM event-specific quantification plasmids.

Results have highlighted the advantages and disadvantages of the use of plasmids as reference materials. Reference GM plasmids provided convenient and reliable positive controls for GM PCR tests. Soya and oilseed rape GM event and endogenous control plasmids have been assessed for quantification purposes. Oilseed rape GM quantification (in seed mixtures) was found to be more problematic than soya due to variable inhibition of real-time PCR by different DNA extracts. We have found that absolute quantification of GM DNA using plasmids as standards in real-time PCR *without other reference materials* may not be possible. Problems were encountered in quantification and matrix-matching errors which may not easily be removed. However, when plasmid standards were used in conjunction with plant tissue reference materials, (at least periodically for their accurate calibration) they can provide quantification results equivalent or better than other reference standards. The complex issue of matrix matching of reference standards (including plasmids) requires further investigation which is currently underway through EU funded projects.

Introduction

Since the inception of this project, the issue of availability of GM quantitative and qualitative standards has become even more pressing. Recently introduced legislation (EC1829/2003 and EC1830/2003) requires EU member states to test for GM presence in non-GM seeds and foodstuffs and enforce labelling when EC thresholds are exceeded. This legislation is likely to result in a rapid increase in the number of EU authorised GM events, in addition the number of world-wide GM events, as yet unauthorised in the EU, continues to grow. The need for adequate reference standards is therefore twofold: firstly qualitative PCR standards are required to act as positive controls for the identification of authorised and unauthorised GM events; whilst secondly quantitative standards are required to construct standard curves for GM DNA and total plant DNA to enable the absolute quantification of GM, thereby ensuring compliance with labelling requirements for authorised GM events. Plasmids have several theoretical advantages as reference materials and quantification standards:

- Plasmid purity and quantification is in theory more simple to control than CRMs which rely on plant material
- Plasmids can be prepared much more cheaply per test application because they are used in very low concentration to simulate the copy numbers of target sequences in genomic DNA
- A single plasmid preparation can be used as a 'gold standard' because its dilutions will provide a very large number of tests (e.g. 1µg of average sized plasmid is enough for approximately 1×10^8 tests of 5% equivalent GM)

However, disadvantages also exist:

- Their massive effective copy number when undiluted is a contamination risk for other GM tests
- It cannot be known that assays on pure plasmids will give the same signal as assays on DNA from other matrices which contain other DNA and contaminants

Plasmids have recently become much more widely used as reference materials. Many research and GM testing laboratories have taken up their use due to an absence of CRMs for the majority of GM events released worldwide. Although common events are represented by CRMs (e.g. MON810 maize, Bt maize, Roundup-Ready Soya (RRS)), many species and rarer events are not available. Currently no commercially available CRMs are available for oilseed rape (OSR). Two plasmids are commercially available as quantitative standards; developed by NipponGene, Japan and are distributed in the EU by Diagenode, Belgium (<http://www.diagenode.com/Environement/Environment.php>), maize and RR soya only are available. At the time of writing we are not aware of any published validation studies of these plasmids. Many independent research groups have now investigated real-time PCR (RT-PCR) quantification of GM using plasmids as reference standards, for several species, including Soya (Huang & Pan, 2005; Terry *et al.*, 2002; Taverniers *et al.*, 2004; and Pardigol *et al.*, 2003), maize (Taverniers *et al.*, 2005; Nielsen *et al.*, 2004; Hernandez *et al.*, 2003; Collonier *et al.*, 2005; Weighardt *et al.*, 2004 and Huang & Pan, 2004), OSR (Taverniers *et al.*, 2005 and Block & Schwarz, 2003) and potato (Rho *et al.*, 2004). All of these have reported that plasmids perform at least as well, and often better than quantification standards made from genomic DNA (either dilutions of

100% GM DNA or extractions of w/w CRMs). With minor differences between workers, the above plasmids have been produced in the same way. PCRs were performed on GM material using GM-specific primers. The PCR products were then cloned into commercially available PCR plasmid vectors (e.g. pGEM, Promega), purified and quantified. Finally, the plasmid was diluted either in water or buffer or a background solution of non-target DNA. There are three critical steps in this process: 1) specific cloning of the desired GM sequence; 2) accurate quantification and dilution of the pure plasmid; and 3) ensuring that the plasmid standards behave in a similar manner to their equivalent of genomic DNA (commutability - Taverniers *et al.*, 2005). The first can be simply controlled by reliable screening of clones and their sequencing to confirm their identity. The second is purely a matter of using accurate physical quantification methods, such as fluorometry and technical expertise in handling solutions. The third is not simple to control as it is not possible to predict the effect of genomic DNA or other constituents of the DNA extract on the efficiency of the RT-PCR. Taverniers *et al.*, (2005) have established that this 'matrix matching' is an important factor despite their genomic DNA and plasmid calibration curves not differing significantly. Matrix matching effects and other factors such as dilution errors are compounded by the requirement for two separate assays for any RT-PCR quantification of GM. A GM quantification and an endogenous DNA quantification (total target organism DNA) must be performed. If the assays have different efficiencies in different matrices or plasmids are quantified or diluted with different errors then large inaccuracies could arise in the final quantification calculations. Mattarucchi *et al.*, (2005) have attempted to reduce these errors by placing the endogenous and GM sequence on the same tandem plasmid. While this reduces errors from plasmid dilution and quantification it does not remove errors due to matrix matching requirements.

Methods

The following gives a brief summary of methods developed for this project. Detailed protocols are given in Appendix 1.

Materials

Due to the unavailability of plasmids reference materials for most GM events, it was important within the scope of this project to provide the methods to make and test in-house plasmids. Such methods, once validated can be used to make new standard plasmids when they are required with the shortest possible delay. To this end, in order to fully assess new methods and plasmids, plant materials have been selected from the major GM crops: soybean, maize and OSR.

Plant material was grown in glasshouses from seed previously identified as containing the GM event of interest. DNA from adult plants was extracted and tested with PCR and RT-PCR to determine their GM genotype (i.e. hetero- or homozygous) using methods previously described in Allnutt *et al.*, 2005. Therefore seed of known GM genotype was used for DNA extractions and % OSR GM seed calibration standards.

For OSR, four series of seed-based GM concentration standards were made using homozygous RF3 and MS8 seed diluted in two different varieties of non-GM seed, 'Tambora' and 'Acrobat'. The Tambora seed was from a certified seed lot and was

coated with seed preservative. The Acrobat seed was a sample from a harvested field and was uncoated. Dilutions were made in a total of 1000 seeds by mass where numbers were greater than 100 and by individual seed counts where numbers were less than 100. Single seed mass was determined as the 100 seed mass / 100 independently for each variety and GM line used.

Plasmid Classes

We have identified four classes of plasmid as necessary for GM event identification and quantification:

1. Generic GM sequence plasmids. Containing a commonly used GM sequence, such as 35S promoter. Such plasmids can be used as standards across different species and events.
2. Event specific plasmids. Containing unique sequence which flanks a GM event and plant DNA insertion site or a unique sequence within the GM event. At least one of this class will be required for every GM event to be studied.
3. Endogenous control plasmids. Containing specific (but not necessarily exclusive) sequence of the target species' nuclear genome which is present in a known, low copy number. These plasmids are essential to allow the absolute quantification of the number of GM events present per total number of plant genomes, and therefore the %GM DNA. One plasmid of this class is required for each crop species.
4. Contaminant identification. Non-GMO viruses and bacteria have the potential to contaminate samples and produce false positive tests. Tests to identify the presence of such contaminants require plasmids as positive controls.

PCRs and Plasmid Production

Each of the plasmids was produced by PCR from DNA extracted from plant tissue containing the target GM event. Table 1 gives the details of the plasmids made and the originating tissue of the DNA. Primers for PCR were designed either from published sequences (references in Table 2) or from sequence obtained from suppression PCR products (Allnutt *et al*, 2005). Following PCR of the target sequence, products were ligated into pGEM-T easy or pGEM-T (Promega). DH5 α competent cells were transformed by the PCR ligations and plated onto ampicillin agar with blue / white selection. Positive colonies were picked, replicated, and placed directly into PCRs to amplify the plasmid insertion sites (see Allnutt *et al*, 2005 for SOPs). Colonies found to have the expected insertion size were grown on and plasmid purified using standard alkali lysis methods (Appendix 1, SOP1). Purified plasmid was then linearised with either NotI or PstI restriction enzymes and run on a preparative agarose gel. The linear plasmid band was excised from the gel and the DNA extracted to obtain pure linear plasmid DNA. This DNA was then quantified by Pico Green fluorescence (Appendix 1, SOP2).

Plasmids were sequenced using 1 μ l of plasmid in 20 μ l reactions with standard M13 sequencing primers, by and according to methods of University of Dundee Sequencing Service (<http://www.dnaseq.co.uk>). All sequences were identical to that expected on public databases where such information was available, except pSSIIB (see results). All plasmid insert sequences are given in Appendix 2.

The specificity of plasmids for use as positive control references was tested by standard PCR (method given in Allnutt *et al.*, 2005). In a series of reactions containing an amount of plasmid equivalent to 100 ng genomic DNA, the plasmid specific primer pair and primer pairs designed to other plasmids were used.

Table 1. Description of plasmids produced. Details of primer's sequences used to amplify the target are available in Allnutt *et al.*, (2005).

Name	Target	Insert size (bp)	PCR primers	Plasmid Class	DNA source material	Reference
pRRLF	GTS40-3-2 35s-plant junction	359	RR35s1 - RR35sPLT2	Event specific	Soybean GTS40-3-2 leaf DNA	Windels <i>et al.</i> , 2001 and Allnutt <i>et al.</i> , 2005
pLEC3-4	Soya lectin gene	229	LE3 - LE4	Endogenous control	Soybean GTS40-3-2 leaf DNA	Hird <i>et al.</i> , 2003
pMON810	MON810 CryIab gene-plant junction	294	CRY810C - MON810R	Event specific	Maize MON810 5% CRM flour	Hernandez <i>et al.</i> , 2003
pSSIIB	Maize starch synthase II B gene	502	SSIIA - SSIIB	Endogenous control	Maize MON810 5% CRM flour	Huang <i>et al.</i> , 2004
pRF3	RF3 Tg7 - plant junction	681	RF3RF - Tg7t1out	Event specific	Oilseed rape MS8xRF3 seed	Allnutt <i>et al.</i> , 2005
pMS8	MS8 Tg7 - plant junction		MS8RF - Tg7t1out	Event specific	Oilseed rape MS8xRF3 seed	Allnutt <i>et al.</i> , 2005
pOSRA	Brassica mapping project 'A' genome probe	536	OSR2F-OSR2R	Endogenous control	Oilseed rape MS8xRF3 seed	Allnutt <i>et al.</i> , 2005
pCAMVRT	CAMV RT gene	754	CAMVRT1-CAMVRT3	Contaminant	Anonymous CAMV isolation sample	Franck <i>et al.</i> , 1980

Plasmid Copy Number

Initial spectrophotometric quantifications of plasmids preparations were erratic and much higher than expected (results not shown), perhaps due to contaminants from the gel purification procedure. Spectrophotometry requires a relatively large volume of sample which would be difficult and unnecessary to obtain from restriction digestions of plasmid. All further quantification was therefore done fluorometrically using the Pico Green assay (Molecular Probes Inc.).

Using the quantified, purified linear plasmid, dilutions were made of suitable concentration for real time PCR analyses. Typically, real time PCR methods use 50ng of target DNA per reaction (Lipp *et al.*, 2005). Using published 1C genome sizes (Arumuganathan & Earle, 1991) the number of genomes (and therefore target GM sequence copies per ng of nuclear genomic DNA) was calculated for each species. From the concentration of plasmid derived from fluorescence readings the number of copies of plasmid per μ l was calculated and hence the dilution required to give plasmid solutions theoretically equivalent to GM genomic DNA amounts.

Quantification Standards

Plasmid standards were produced in two types: 1) Absolute standards i.e. a single plasmid in solution with a predetermined concentration equivalent to a known amount per reaction of target DNA; and 2) Relative standards, where both GM target and endogenous sequence plasmids of known concentration were mixed to produce solutions equivalent to different % GM genomic DNA solutions.

Absolute genomic DNA standards were made by serial dilution of 100% GM DNA of DNA of known concentration in molecular biology grade water. Relative seed standards were produced by dispensing counts (by mass when over 100) of known genotype (homozygous) full-sib GM seed into non-GM seed. For RR Soya, for which CRMs are available, a single sample of 5% RR Soya DNA was also included in analyses.

Real time PCR

Primer and probe sets were designed with the aid of the Primer Express software (Perkin Elmer). Event-specific probes were designed to span the junction of plant and GM DNA in plasmid (and plant) sequences. Appendix 1, SOP3, gives details of the Taqman RT-PCR methods.

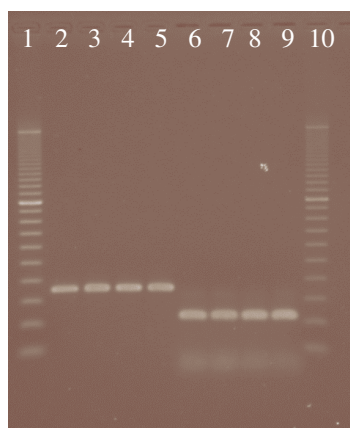
Quantification of GM using RT-PCR has been performed using two different approaches, relative and absolute. For absolute quantification two standard curves were constructed, one for the RT-PCR cycle threshold (Ct) of the GM assay versus log amount of GM target in the reaction; and the other for Ct of plant endogenous assay versus log total amount of plant DNA in the reaction. In this case the proportion of GM to total DNA interpolated from the two curves gives the %GM. The amounts here are expressed in ng DNA per reaction (haploid genome equivalents). Therefore for genomic DNA standards, this is the actual mass of DNA in each reaction, and for plasmid standards it is the equivalent ng of genomic DNA that the amount of plasmid is equivalent to. For relative quantification a single standard curve was plotted of the difference in Ct (Δ Ct) between the endogenous and GM targets versus the log proportion (%) of GM DNA to total plant DNA.

Results and Discussion

Plasmid preparation

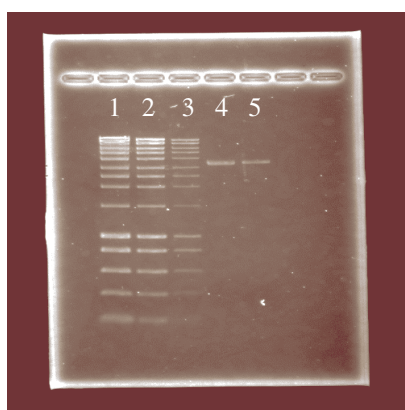
PCRs to obtain plasmid inserts have proved successful using DNA from a range of materials, including certified reference soya and maize flours, and oilseed rape leaves and seeds. Figure 1 shows Roundup-Ready soybean PCR product used for ligation and cloning.

Figure 1. Roundup-Ready soybean (event GTS40-3-2) PCR products used for ligation to plasmids. Lanes 1 and 10 are 100bp marker ladder. Lanes 2-5 are the GM event-plant DNA flanking sequence used in pRRLF. Lanes 6-9 are a portion of the LEC1 gene used as soybean endogenous control in pLEC3-4.



The linearisation and gel purification of plasmids was successful in removing potential concatemeric plasmid forms and bacterial chromosomal DNA, see Figure 2. However, complete removal of bacterial chromosomal DNA from the undiluted plasmid sample may not be possible. This problem is circumvented by the high degree of dilution required (approximately 10^{-5}) in order to make the STD plasmid solutions. The plasmid is therefore at a much higher concentration than any potential contaminants and when diluted further, for production of real-time PCR calibration curves, the bacterial DNA contamination would be far below single copy levels and therefore not able to interfere with GM DNA detection or quantification.

Figure 2. Gel of linearised, purified plasmid pLEC3-4. Lanes 1-3 are size and concentration standards. Lanes 4 and 5 are 5 μ l and 2 μ l of plasmid respectively.



As a preliminary analysis to RT-PCR, plasmids were diluted by factors of ten in water until a theoretical concentration of less than one copy per PCR reaction. Figure 3 shows a gel of PCR products for plasmids, pEP1-2 and pRRLF both of which fail to amplify at between 86 and 8.6 copies per reaction. Note that pEP1-2 was not used in further studies because it was found not to be event-specific (results not shown). Due to the binomial probability of obtaining an exact number of copies sufficient to initiate amplification, this is a reasonable detection limit for the PCR. Fluorometric quantification of plasmids was therefore sufficiently accurate to provide plasmid dilutions in the expected range.

The specificity of PCR primers designed for each plasmid was demonstrated by the amplification of expected products (Figure 4).

Figure 3. Gel showing PCR products from serial dilutions: 86580, 8658, 865, 86, 8.6, 0.08, 0.008 and zero copies per reaction and water control of plasmids pEP1-2 and pRRLF, lanes 2-9 and 10-17 respectively. Lanes 1 and 18 are 100bp size markers. The lower bands are non-specific primer products which increase as the target sequence amount decreases.

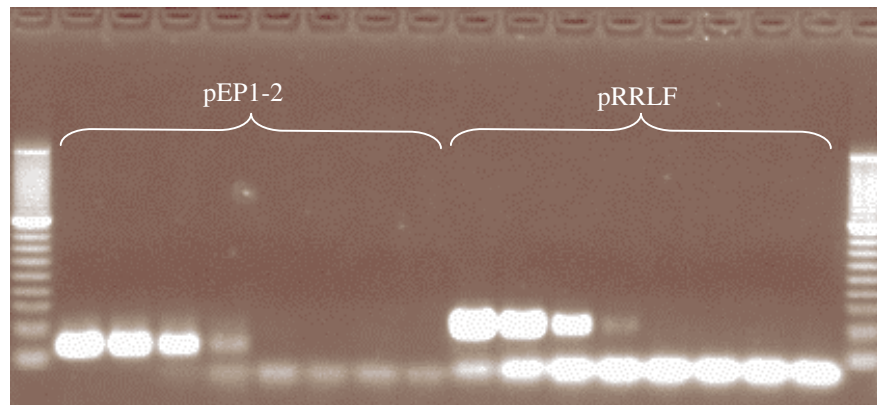
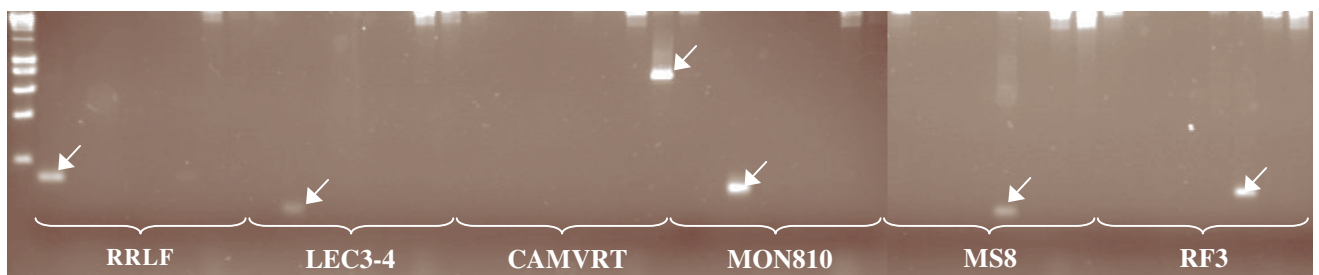


Figure 4. Gel showing PCR products of plasmid specificity test. Each primer set was tested with: pRRLF, pLEC3-4; pMON810, pSSIIB, pMS8, pRF3, pOSRA and pCAMVRT (each primer set lanes 1-8 respectively). Arrows indicate expected PCR product for each primer set.



Real-Time PCR

Known soya and OSR DNA standards were assayed using real-time PCR to assess the accuracy of plasmid standard curves. Additional assays (not shown) using maize showed that SSIIB Ct values were much higher than expected. Examination of the pSSIIB plasmid sequence showed that it contained a 288 bp insert (intron) not indicated on GenBank databases. This resulted in an amplicon of 288bp larger than anticipated when the primers and probe were designed to the database sequence. The large amplicon size accounted for the relatively low reaction efficiency.

For RR soya, the two assays, GM event-specific (RRLF) and endogenous gene (LEC3-4), were used with absolute standard series of plasmid and genomic DNA (isolated from 100% RR soya seeds and diluted in water). The amounts (in ng) per reaction were calculated from the dilution factor of stock solutions, the concentrations of which were determined by fluorometry as detailed above. Standard curves for plasmids pRRLF and pLEC3-4 and genomic DNA dilutions gave clear linear relationships to log target DNA amount as expected (see Tables 2 and 3 and Figures 5 and 6) with good correlation coefficients (R^2 values shown on graphs). The efficiencies of reactions were shown by the slope of the curves which were close to ideal (-3.3) in all cases.

Important differences are evident in the results between genomic DNA and plasmid standards. The concentration of pRRLF appears to have been underestimated due to an unknown error, possibly in dilutions. This is evident from the larger intercept value for pRRLF compared to the genomic DNA RRLF curve (Figures 5 and 6 respectively). Table 3 shows the calculation of target DNA (ng) in the genomic standards using the plasmid standard curve: pRRLF values differ by a mean factor of 1.9; and pLEC3-4 by a lower factor of 1.1. Although gradients between genomic DNA and plasmid curves were similar, they were not identical and due to the log transformation of the mass (x) axis, this can lead to large differences in calculated mass readings from unknown samples even when the amount of DNA is re-calibrated.

In order to examine the dynamic range of the real-time method and the accuracy of the calibrations, triplicate samples of dilutions of DNA isolated from 5% RR soya CRM were tested (Table 4). Across the range of the experiment no clear trends in deviation of calculated % GM were observed. However, Table 4 shows that different values were obtained from plasmid and genomic DNA calibrations, with the latter being considerably more accurate (genomic DNA %CV = 10.98%, where CV calculated as % variation of deviation from expected value). When pRRLF standards were adjusted using the genomic DNA calibration to re-calibrate the highest plasmid standard (all those below following the expected dilution factor), plasmid standard calculated values were improved (%CV = 32.5). This result suggests that an absence of matrix matching for the plasmid standards may be responsible for their inferior accuracy to the genomic DNA standards used here, as evident from the different gradient of plasmid curves. Dilution of the plasmid in non soya plant DNA may have improved these results.

These results also show that errors can be introduced in plasmid quantification that may be of much higher orders than those for genomic DNA. This is likely to be due to the very high dilution factor required to obtain useful plasmid standards from

solutions that have a measurable DNA content (typically 10^5 to 10^7). Caution must therefore be taken when making such dilutions. The dilution of higher concentration plasmid may be more prone to errors (due to clumping or adsorption of plasmid) than the lower concentrations, because no deviation from linearity or 'steps' were observed in the dilutions within the range of the plasmid standard curve.

Table 2. Results of Taqman real-time PCR calibration of using pRRLF and pLEC3-4 absolute quantification standards. Mean Ct and standard deviation of duplicate readings are shown. Zero ng (water) controls always gave undetermined Ct values.

Plasmid	ng	Mean Ct	sd
pRRLF	100	21.76	0.07
	50	22.70	0.03
	10	25.38	0.18
	5	26.21	0.27
	1	28.78	0.13
	0.5	29.40	0.21
	0.1	32.44	0.18
pLEC3-4	100	25.30	0.19
	50	26.18	0.03
	10	28.74	0.01
	5	29.64	0.22
	1	31.46	na
	0.5	32.80	0.24
	0.1	35.10	0.28

Table 3. Results of Taqman real-time PCR calibration of using RR soya genomic DNA quantification standards. Mean Ct and standard deviation of duplicate readings are shown. Zero ng (water) controls always gave undetermined Ct values.

RT PCR assay	ng	Mean Ct	sd	ng (Plasmid)
RRLF	46	25.57	0.11	28.55
	10	27.68	0.01	7.13
	5	29.09	0.19	2.84
	1	31.72	0.40	0.51
	0.5	32.68	0.43	0.27
	0.1	36.51	na	0.02
	LEC	46	25.37	0.02
10		27.73	0.11	9.73
5		28.54	0.24	5.50
1		31.38	0.16	0.73
0.5		32.10	0.14	0.44
0.1		35.43	0.15	0.04

Table 4. Real-time PCR results for triplicate dilutions of 5% RR soya CRM tested using plasmid and genomic DNA calibration standards. For each sample the amount of target (ng) was interpolated from the standard curve and for the genomic and adjusted plasmid curves, the %CV calculated. The adjusted plasmid curve was made by recalculating the highest standard using the genomic standard curve.

Samples dilution	Calc. using plasmids			Calc. using genomic				Calc. using adjusted plasmids			
	ng rr	ng lec	%	ng rr	ng lec	%	%CV	ng rr	ng lec	%	%CV
1 in 5	5.51	1271.07	0.43	28.20	492.67	5.72	10.23	19.86	685.10	2.90	29.71
	4.86	999.15	0.49	25.23	399.13	6.32	18.69	17.53	538.53	3.26	24.68
	4.35	957.60	0.45	22.84	384.58	5.94	13.30	15.68	516.14	3.04	27.76
1 in 10	2.76	630.64	0.44	15.26	266.88	5.72	10.16	9.96	339.91	2.93	29.27
	2.27	587.53	0.39	12.81	250.85	5.11	1.49	8.18	316.68	2.58	34.20
	2.71	535.87	0.51	15.00	231.45	6.48	20.92	9.76	288.83	3.38	22.90
1 in 20	1.38	365.62	0.38	8.21	165.67	4.96	0.60	4.96	197.07	2.52	35.11
	0.88	363.04	0.24	5.52	164.64	3.35	23.31	3.17	195.68	1.62	47.78
	1.60	430.28	0.37	9.39	191.02	4.92	1.16	5.77	231.92	2.49	35.52
1 in 40	1.04	178.85	0.58	6.39	88.64	7.21	31.20	3.74	96.40	3.88	15.85
	0.63	213.48	0.29	4.10	103.48	3.96	14.73	2.27	115.06	1.97	42.83
	0.86	237.40	0.36	5.42	113.55	4.78	3.17	3.11	127.96	2.43	36.33
1 in 80	0.42	119.46	0.35	2.83	62.28	4.55	6.35	1.50	64.39	2.33	37.78
	0.31	79.23	0.39	2.18	43.48	5.01	0.14	1.12	42.71	2.61	33.78
	0.37	149.84	0.25	2.58	75.92	3.40	22.63	1.35	80.76	1.67	47.08
1 in 160	0.12	47.93	0.25	0.94	28.02	3.35	23.32	0.43	25.83	1.68	47.02
	0.25	14.69	na	1.82	9.96	na	na	0.91	7.92	na	na
	0.20	46.26	0.42	1.45	27.16	5.33	4.63	0.70	24.94	2.82	30.79
1 in 320	0.12	24.99	0.47	0.92	15.85	5.79	11.13	0.42	13.47	3.13	26.45
	0.13	29.62	0.44	1.01	18.39	5.51	7.20	0.47	15.96	2.95	28.95
	0.12	28.38	0.43	0.95	17.72	5.36	5.11	0.44	15.30	2.87	30.18
1 in 640	0.03	7.14	0.40	0.26	5.30	4.90	1.43	0.10	3.85	2.65	33.26
	0.04	8.40	0.53	0.39	6.11	6.36	19.22	0.16	4.53	3.54	20.62
	0.03	7.99	0.42	0.30	5.85	5.16	2.33	0.12	4.31	2.81	31.04
mean value			0.40			5.18	10.98			2.70	32.56

Figure 5. Plasmid standard curves for pLEC3-4 (squares) and pRRLF (diamonds).

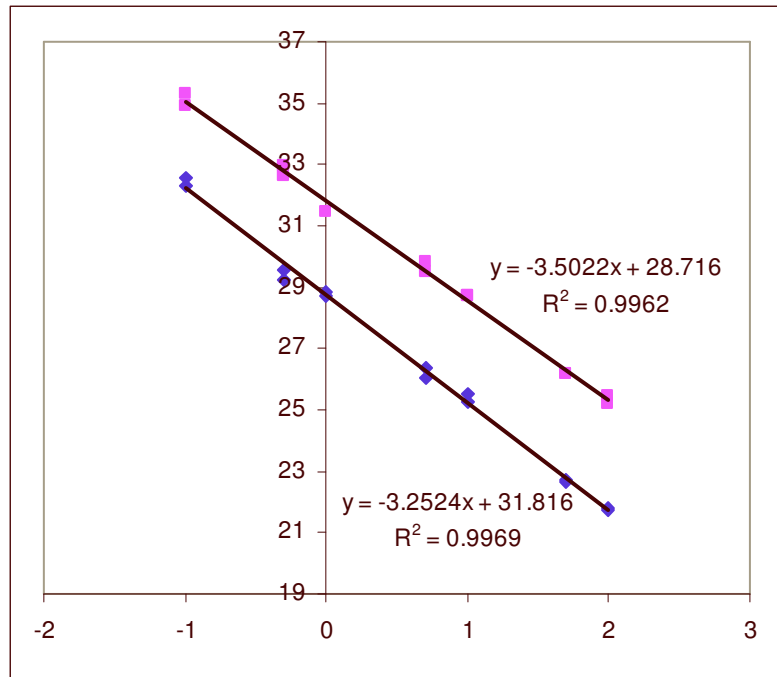
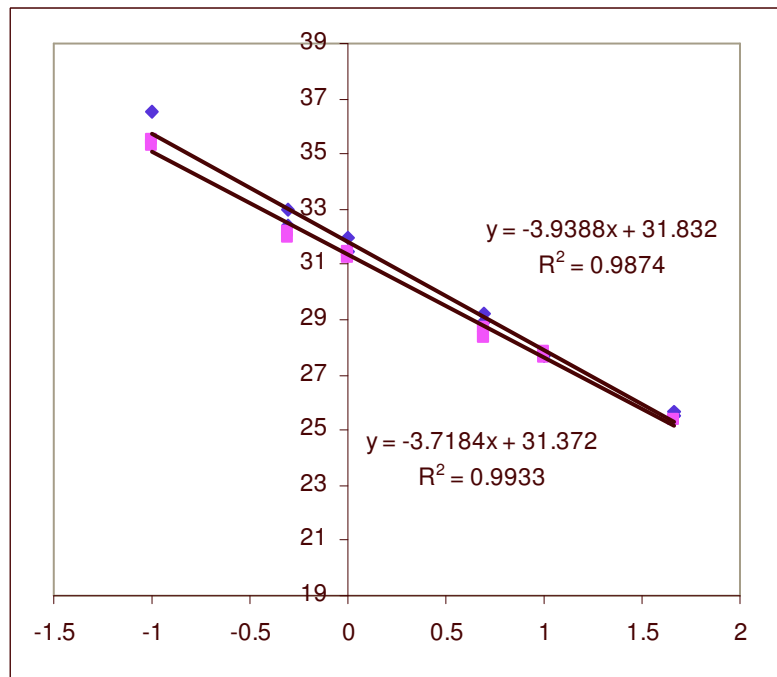


Figure 6. . Genomic DNA standard curves for LEC3-4 (squares) and RRLF (diamonds).



Real time PCRs were performed with three OSR assays: GM events specific assays for MS8 and RF3; and the endogenous gene assay for the OSR2A sequence. The ΔC_t relative quantification method was used. Four series of seed-based GM concentration standards were made using homozygous RF3 and MS8 seed diluted in two different varieties of non-GM seed as detailed above. Ct was determined in triplicate for each plasmid standard series (pMS8 mixed with pOSR2A; and pRF3 mixed with pOSR2A to give equivalent %GM DNA solutions).

Table 5 summarises the OSR results. For each seed standard dilution series with known concentration, the plasmid standard curve was used to calculate %GM DNA values. Given the homozygous genotypes of the GM seed, it was expected that the plasmid calculated values should be equivalent to the actual % GM seed. This was not the case, and several differences are evident between different seed dilutions and between the two GM events.

For the MS8 event and plasmid, seed dilution G gave promising results with mean CV = 21.23%, well within the ENGL recommended value of 25%. The same assay however, with seed dilutions E, gave values which differed from those expected by a factor of two (mean CV = 77.97%). It would seem that this error factor of almost exactly two could be explained simply by a discrepancy in the copy number (zygosity) of the OSR2A sequence between the G non-GM seed (Acrobat) and the E non-GM seed (Tambora). However, this cannot be the case because this factor of two was not observed between the two varieties in the RF3 assay.

Table 5. Summary of OSR GM event specific plasmid results. '%' gives the actual % GM DNA in each standard. The mean Δ Ct of triplicates is given. Outlier results (>factor of 3 deviation from linear regression) were excluded. The plasmid standard curve (Figures 7 and 8) were used to calculate observed real-time PCR % GM DNA for each event. The calibration factor gives the coefficient by which the observed value deviated from expected. All bold values are means for that column. The adjusted % is the product of the plasmid value and the calibration factor. G and D = Tambora and E and B = Acrobat non-GM seed used for dilutions.

Standard	%	Mean dCt	% Calculated by		Calibration factor	Adjusted % calculated by	
			plasmid	%CV		plasmid	%CV
G Seed MS8	5	6.23	6.53	16.38	1.31	5.88	12.49
	2	7.21	3.31	38.78	1.66	2.98	34.72
	1	8.77	1.13	3.06	1.13	1.02	1.27
	0.5	na	na	na	na	na	na
	0.2	11.81	0.14	26.76	0.70	0.13	26.12
	0.1	12.63	0.08	21.15	0.78	0.07	21.02
				21.23	1.11		
E seed MS8	5	5.48	10.98	84.57	2.20	5.23	3.23
	2	7.14	3.48	52.33	1.74	1.66	12.12
	1	7.39	2.91	135.06	2.91	1.39	27.27
	0.5	9.00	0.96	65.05	1.92	0.46	6.06
	0.2	10.37	0.37	60.10	1.85	0.18	8.42
	0.1	11.30	0.20	70.71	2.00	0.10	3.37
				77.97	2.10		
D seed RF3	5	6.34	14.19	129.97	2.84	3.66	18.99
	2	7.45	6.66	164.76	3.33	1.72	10.02
	1	8.77	2.75	123.74	2.75	0.71	20.59
	0.5	9.50	1.66	164.05	3.32	0.43	10.21
	0.2	9.69	1.47	449.01	7.35	0.38	63.24
	0.1	11.75	0.37	190.92	3.70	0.10	3.28
				203.74	3.88		
B seed RF3	5	5.68	22.03	240.84	4.41	5.40	5.65
	2	7.36	7.09	180.10	3.55	1.74	9.24
	1	7.75	5.47	316.08	5.47	1.34	24.09
	0.5	na	na	na	na	na	na
	0.2	10.75	0.72	183.85	3.60	0.18	8.32
	0.1	11.88	0.34	169.71	3.40	0.08	11.79
				218.11	4.08		

Figure 7. Standard curve for ΔC_t (MS8 - OSR2A) vs. log relative % plasmid concentration (pMS8 mixed with pOSR2A).

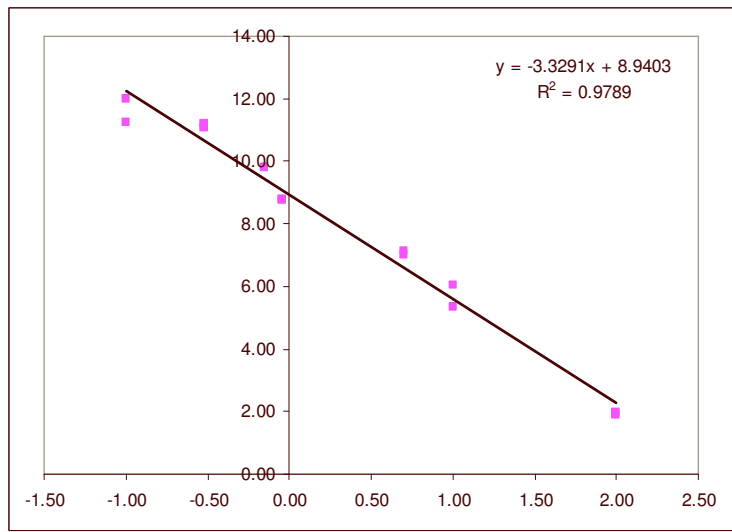
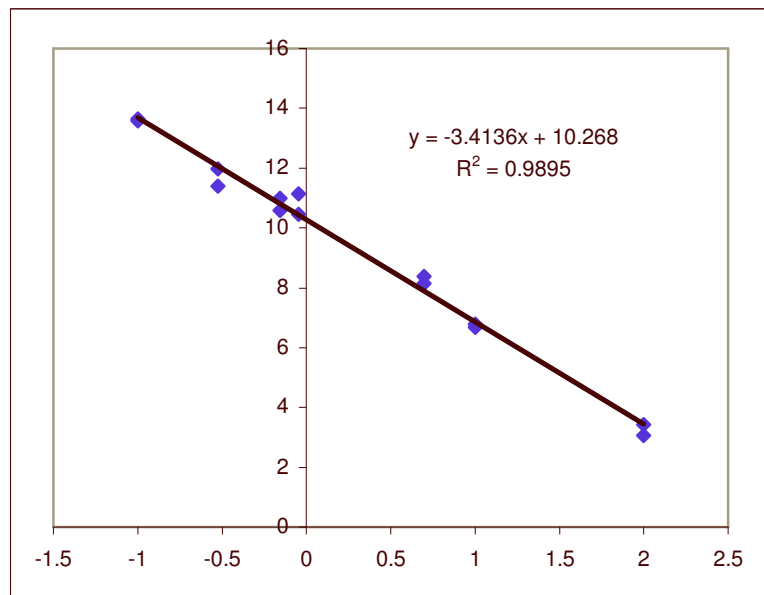


Figure 7. Standard curve for ΔC_t (RF3 - OSR2A) vs. log relative % plasmid concentration (pRF3 mixed with pOSR2A).



For both seed dilution series used with the RF3 assay, the plasmid standard curve overestimated % GM by a factor of approximately four but unlike MS8, this factor was similar for both Tambora and Acrobat series. The reasons for these discrepancies are obscure and could be due to a number of causes. There are many stochastic errors, such as plasmid dilution accuracy which could have contributed but the variation in errors between the different seed dilutions would point to several confounding factors. Tambora seed was treated with a preservative coating, which Acrobat was not. It is possible that this treatment or some other constituent in the seed samples acted to inhibit RF3, MS8 and / or OSR2A assays differentially. Whichever reason is true, the results show that without thorough examination, commutability of plasmid standards to 'real' samples cannot always be assumed. Table 5 also shows the application of a 'calibration factor' to the plasmid calculated results, which is the mean deviance between the plasmid curve calculated value and the true % GM seed. When this was performed for each seed dilution series, very low %CV (within ENGL limits) were obtained. Such calibration factors are often applied to allow for differences in genotype between standards and samples and they have been suggested good practice for regular calibration of plasmid standards against 'real' samples to ensure commutability. However, it is evident from these results that sometimes a calibration factor obtained from one set of OSR seeds may not be applicable to another (a found in the MS8 assay). A consistency of calibration factor on a wide array of samples must therefore be demonstrated (as is the case for the RF3 assay for two seed varieties - ideally more would need to be examined). These 'matrix matching' problems are also likely to apply to soya. Only one variety and source of non-GM material was available for our RR soya studies, so it was not possible to establish if calibration factors for soya plasmids would vary.

Conclusions

Proper reference materials are essential for interpretation of qualitative and quantitative PCR assays for GM events. This work has shown that where reference materials derived directly from reference plant tissues do not exist, are not available or are impractical for frequent use, plasmids can quickly be produced and used for this purpose. When used at appropriate dilution to avoid contamination issues and give realistic copy numbers per reaction, plasmids provided an excellent and economic alternative to plant DNA extractions for positive control material. Their use as positive controls is likely to increase for method validation, quality control and method development: in particular plasmid targets provide a convenient means to produce solutions to test detection limits and error rates of qualitative and quantitative PCRs.

When using plasmids for GM quantification caution must be applied and several measures undertaken to avoid potentially large errors. The data presented have shown that in some cases and with proper treatment, plasmids can provide standards equivalent or more accurate than genomic DNA extractions. However, this can only be achieved when the following measures are taken:

- Accurate and precise quantification and dilution of plasmid standards must be ensured. DNA quantification can be problematic and confounded by many contaminants and parameters, e.g., salt concentration, pH. Double-stranded

specific fluorometric assays (which are not affected by protein or RNA) are currently most reliable. It may further be necessary to assay new plasmid dilutions against 'gold standard' dilutions which are made much less frequently and provide a common reference for larger numbers of plasmid working dilutions.

- Calibration of plasmids against standards made from the GM matrix to be tested must be undertaken regularly. If commutability is not tested in this way, slight changes in non-GM matrix (such as seed variety as shown in this study) can increase quantification errors.
- It may not be possible to ensure a plasmid standard series will give a 1:1 result with plant GM samples because the relative efficiency and copy number of GM target and endogenous control sequence may not be known or may be variable. In this case, the use of a calibration factor may be appropriate. However, the variation of the calibration factor over the dynamic range of the assay should be determined.

Despite the economic and practical advantages of using plasmids for GM quantification assays it should be noted that they can never be used purely in isolation from the GM plant tissue and non-GM matrix to be tested. In some cases, such as where inhibitory effects are very variable, it may only be possible to use standards prepared from the test matrix itself. This may be prohibitively expensive for routine analyses, but when quantitative results are bordering on specified thresholds or limits then it may be the most advisable approach.

References

- Allnutt, T. R., Roper, K., Thomas, C., Hugo, S., Kerrins, G. & Henry, C. (2005). Detection and Traceability Technologies to Underpin the GM Inspectorate. *Final Report to Defra*.
- Arumuganathan, K. & Earle, E. D. (1991) Nuclear DNA Content of Some Important Plant Species. *Plant Molecular Biology Reporter* 9, 208-218.
- Berdal, K.G. & Holst-Jensen, A. (2001) Roundup Ready (R) soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. *European Food Research and Technology* 213 (6), 432-438.
- Block, A. & Schwarz, G. (2003) Validation of different genomic and cloned DNA calibration standards for construct-specific quantification of LibertyLink in rapeseed by real-time PCR. *European Food Research Technology* 216,421-427.
- Franck,A., Guilley,H., Jonard,G., Richards,K. and Hirth,L. (1980). Nucleotide sequence of cauliflower mosaic virus DNA. *Cell* 21 (1), 285-294 (1980)
- Hernandez, M., Pla, M., Esteve, T., Prat, S., Puigdomenech, P. & Ferrando, A. (2003) A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard (R) based on the 3' -transgene integration sequence. *Transgenic Research* 12 (2), 179-189.
- Hird, H., Powell, J., Johnson, M-L and Oehlschlager, S. (2003) Determination of the Percentage of RoundUp Ready Soya in Soya Flour using Real Time Polymerase Chain Reaction, Interlaboratory Study. *J. AOAC.I* 86,66-71.
- Huang, H.Y. & Pan, T.M. (2004) Detection of genetically modified maize MON810 and NK603 by multiplex and real-time polymerase chain reaction methods. *Journal of Agricultural and Food Chemistry* 52 (11), 3264-3268.

- Huang, C.C. & Pan, T.M. (2005) Event-specific real-time detection and quantification of genetically modified Roundup Ready soybean. *Journal of Agricultural and Food Chemistry* 53 (10), 3833-3839.
- Lipp, M., Shillito, R., Giroux, R., Spiegelhalter, F., Charlton, S., Pinero, D. & Song, P. (2005) Polymerase chain reaction technology as analytical tool in agricultural biotechnology. *Journal of Aoac International* 88 (1), 136-155.
- Mattarucchi, E., Weighardt, F., Barbati, C., Querci, M. & Van den Eede, G. (2005) Development and applications of real-time PCR standards for GMO quantification based on tandem-marker plasmids. *European Food Research and Technology* 221 (3-4), 511-519.
- Nielsen, C.R., Berdal, K.G. & Holst-Jensen, A. (2004) Characterisation of the 5' integration site and development of an event-specific real-time PCR assay for NK603 maize from a low starting copy number. *European Food Research and Technology* 219 (4), 421-427.
- Pardigol, A., Guillet, S. & Popping, B. (2003) A simple procedure for quantification of genetically modified organisms using hybrid amplicon standards. *European Food Research and Technology* 216 (5), 412-420.
- Rho, J.K., Lee, T., Jung, S.I., Kim, T.S., Park, Y.H. & Kim, Y.M. (2004) Qualitative and quantitative PCR methods for detection of three lines of genetically modified potatoes. *Journal of Agricultural and Food Chemistry* 52 (11), 3269-3274.
- Taverniers, I., Van Bockstaele, E. & De Loose, M. (2004) Cloned plasmid DNA fragments as calibrators for controlling GMOs, different real-time duplex quantitative PCR methods. *Analytical and Bioanalytical Chemistry* 378 (5), 1198-1207.
- Taverniers, I., Windels, P., Vaitilingom, M., Milcamps, A., Van Bockstaele, E., Van den Eede, G. & De Loose, M. (2005) Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176, and GA21 maize and transgenic GT73 canola. *Journal of Agricultural and Food Chemistry* 53 (8), 3041-3052.
- Terry, C.F., Shanahan, D.J., Ballam, L.D., Harris, N., McDonnell, D.G. & Parkes, H.C. (2002) Real-time detection of genetically modified soya using lightcycler and ABI 7700 platforms with TaqMan, Scorpion, and SYBR Green I chemistries. *Journal of Aoac International* 85 (4), 938-944.
- Weighardt, F., Barbati, C., Paoletti, C., Querci, M., Kay, S., De Beuckeleer, M. & Van den Eede, G. (2004) Real-time polymerase chain reaction-based approach for quantification of the pat gene in the T25 Zea mays event. *Journal of Aoac International* 87 (6), 1342-1355.
- Windels, P., Taverniers, I., Depicker, A., Van Bockstaele, E. & De Loose, M. (2001) Characterisation of the Roundup Ready soybean insert. *European Food Research and Technology* 213 (2), 107-112.

Appendix 1. Standard Operating Procedures.

The following SOPs constitute detailed methods recorded for quality control purposes within the Central Science Laboratory in the context of the project, "Plasmid Standards for Real Time PCR and GM Enforcement Testing". They should not be interpreted as procedures formally approved by Defra.

SOP 1. Plasmid isolation, alkali lysis method.

Reagents:

- Luria-Bertaini (LB) broth plus 50 µg/ml ampicillin
- Lysis buffer: 25 mM Tris-HCl, pH 8; 10 mM EDTA; 50mM glucose
- 0.4 N NaOH
- 2% SDS
- Potassium acetate (Kac) solution: 60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; and 38.5 ml molecular biology grade water
- TE buffer: 10 mM Tris-HCl, pH 8; 1 mM EDTA

Protocol:

1. Pick one DH5α colony (containing plasmid of interest) and inoculate 100 ml LB broth plus 50 µg/ml ampicillin in a 250 ml conical flask. Incubate overnight with shaking at 37°C. Plasmid yield can be improved if necessary by incubation until log growth phase (usually 4-6 hours).
2. Decant LB culture equally into two 50 ml Oakridge tubes. Centrifuge 5000 × g for 5 minutes at 4°C.
3. Discard supernatant and invert tubes on tissue to drain excess broth from the cell pellets.
4. Resuspend pellets in 2 ml lysis buffer.
5. Add 4 ml of mixed 1:1, 0.4 N NaOH and 2 ml 2% SDS solutions. Mix suspension gently by inversion. Incubate room temperature 5 minutes.
6. Add 3 ml Kac solution, mix gently by inversion.
7. Centrifuge 12000 × g for 10 minutes, room temperature.
8. Transfer supernatant, avoiding any solids to new 50ml Oakridge tube.
9. Add 5 ml chloroform:isoamylalcohol (24:1), mix vigorously.
10. Centrifuge 12000 × g for 10 minutes, room temperature.
11. Remove upper aqueous phase and transfer to new tube. Repeat steps 9-11.
12. Add 8 µl isopropanol, mix and incubate for 20 minutes, -20°C.
13. Centrifuge 12000 × g for 10 minutes.
14. Decant and discard supernatant. Dry pellets by dabbing carefully with clean tissue to remove droplets of isopropanol.
15. Dissolve each DNA pellet 500 µl TE, combine into one 1 ml solution in 1.5 ml microcentrifuge tube, label and store at -20°C.

SOP 2. Plasmid quantification using PicoGreen[®] dsDNA reagent.

Reagents:

- PicoGreen dsDNA quantification kit (Molecular Probes Inc. part: P-7589): PicoGreen reagent; Lambda DNA standard, 100 µg/ml
- TE buffer: 10 mM Tris-HCl, pH 8; 1 mM EDTA

Specific equipment:

- Turner Picofluor handheld fluorometer
- Semi-micro cuvettes (Fisher Scientific, UK, part: FB55147)

Protocol:

1. Make up a 1:200 dilution of Pico Green from the stock solution in TE buffer. Ensure sufficient volume for all assays.
2. Make up 5 × 1.5 ml microcentrifuge tubes containing different DNA standards by adding 2 µg/ml λ DNA standard (made by 1:50 dilution of 100 µg/ml Lambda DNA included in PicoGreen kit), TE and PicoGreen reagent in the following quantities:

ng/ml of DNA	λ DNA 2 µg/ml (µl)	TE (µl)	1:200 PicoGreen (µl)
1000	250	0	250
500	125	125	250
200	50	200	250
50	12.5	237.5	250
0	0	250	250

3. Mix each tube well and then briefly centrifuge.
4. Use one 1.5 ml microcentrifuge tube for each DNA sample to be tested and add the following:

DNA of unknown concentration (µl)	TE (µl)	PicoGreen (µl)	Total Volume (µl)
10	240	250	500

5. Mix each tube well, then briefly centrifuge.
6. Pipette 500 µl from each tube into a separate cuvette.
7. Switch on the fluorometer. Ensure screen reads "0.000 BLUE". Follow Picofluor user manual for taking readings of each standard and sample.
8. Construct a standard curve for DNA standards and use to calculate unknown samples' DNA concentration, including dilution factor of 50 × in calculation. If calculated values are above 1000 ng/ml or below 50 ng/ml use new appropriate dilution of sample DNA and re-quantify.

SOP 3. Real-time PCR.

Equipment:

ABI 770 or 7900 sequence detection system (SDS).
96 well PCR plate (ABgene, AB1100).

Reagents:

ABsolute QPCR ROX 2 × mix (AB gene, UK, part: AB-1138)

Primer and probe sets (Appendix 2)

Protocol:

1. Prepare reaction mastermix per reaction as follows:

Total volume per PCR = 25 µl

Reagent	Stock Concentration	Volume (µl)	Final Concentration
ABgene QPCR readymix	2X	12.5	1X
primer 1	5 µM	2.5	0.5 µM
primer 2	5 µM	2.5	0.5 µM
probe	5 µM	2.0	0.4 µM
Molecular biology grade water	-	0.5	-

2. Add 20 µl of mastermix to each plate well.

3. Add DNA sample (approximately 50 - 100 ng) in 5 µl. Seal the plate and place in SDS.

4. Use following run parameters:

i) Initial 95 °C, 15 min.

ii) 95 °C, 1 min.

iii) 60 °C, 1 min.

Repeat (ii) to (iii) for 45 cycles.

On completion of run, ensure analysis is carried out with baseline cycles set to 5 and 15 and the cycle threshold to 0.2 for all runs to allow for direct comparison of Ct values.

Appendix 2. Plasmid insert sequences

Position and orientation of primers and TaqMan probes are indicated. Probes are marked in dashed lines. Vertical lines through sequences mark the GM event -plant DNA junction where relevant. Note that the cloned PCR product in each case also carries a 3' adenosine residue.

pLEC3-4 insert sequence:

```
ATTGTGACCTCCTCGGGAAAGTTACAACCTCAATAAGGTTGACGAAAACGGCACCCCAAAA
    10          20          30          40          50          60
TAACACTGGAGGAGCCCTTTCAATGTTGAGTTATTCCAACCTGCTTTTGCCGTGGGGTTTT
                                LecF
CCCTCGTCTCTTGGTCGCGCCCTCTACTCCACCCCATCCACATTTGGGACAAAGAAACC
    70          80          90          100-----110-----120
GGGAGCAGAGAACCAGCGCGGGAGATGAGGTGGGGGTAGGTGTAAACCCCTGTTTCTTTGG
                                LecP
GGTAGCGTTGCCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCTGACACAAAA
    130          140          150          160          170          180
CCATCGCAACGGTTCGAAGCGGCGAAGGAAGTTGAAGTGAAGATACGGGGACTGTGTTTT
                                LecR
AGGCTTGCAGATGGGCTTGCCTTCTTTCTCGCACCAATTGACACTAAGCC
    190          200          210          220          230
TCCGAACGTCTACCCGAACGGAAGAAAGAGCGTGGTTAACTGTGATTTCGG
```

pRRLF insert sequence:

```
GATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCT
    10          20          30          40          50          60
CTATCACCCCTAACACGCAGTAGGGAATGCAGTCACCTCTATAGTGTAGTTAGGTGAACGA
                                rrlfF
TTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGTGCTCCTCGTGGGTGGGGTCCATC
    70          80          90          100          110          120
AACTTCTGCACCAACCTTGCAGAAGAAAAAGGTGCACGAGGAGCACCCACCCAGGTAG
                                rrlfP
TTTGGGACCACTGTGCGCAGAGGCATCTTCAACGATGGCCTTTCCTTTATCGCAATGATG
    130          140          150          160          170          180
AAACCCTGGTGACAGCCGTCTCCGTAGAAGTTGCTACCGGAAAGGAAATAGCGTTACTAC
                                <GM plant>
GCATTTGTAGGAGCCACCTTCCCTTTCCATTTGGGTTCCCTATGTTTATTTTAACTGTGA
    190          200          210          220          230          240
CGTAAACATCCTCGGTGGAAGGAAAGGTTAAACCCAAAGGGATACAAATAAAATTTGGACAT
                                rrlfR
TGTATGATCTTATTTTGAATGAAATGCAATAAGTTATTTCTAGTAAAAAAAAATAACAT
    250          260          270          280          290          300
ACATACTAGAATAAAACTTACTTTACGTTATTCAATAAAGATCATTTTTTTTTTATTTGTA
TTGATAGAAACAAATTAAGCATGCAAAAATAACTCATTAGCATCGGTTAAATTGAAGG
    310          320          330          340          350          360
AACTATCTTTGTTTAAATTTTCGTACGTTTTTATTGAGTAATCGTAGCCAATTTAACTTCC
```

pSSIIB insert sequence. Note sequence in lowercase italics is an intron not indicated in original database entry:

```
TGGTACAAGCGGAAGCAGCAGTAGCGTGAGGCATCCCCATGCCGGGGCAATCTTCCCT
    10          20          30          40          50          60
ACCATGTTTCGCCCTTCGTGCTCATCGCACTCCGTAGGGGTACGGCCCCCGTTAGAGAAGGA
CGTCGTGCGCTTTTTCTCCTCCCCGTCGCTCCTCCTCGCCGCGGCAGGCGGGGCAGTG
```

70 80 90 100 110 120
GCAGCAGCCGAAAAGAGGAGGGGCAGCGCAGGAGGAGCGGCGCCCGTCCGCCCCGTAC
TGGGTGCTGCTCTGCGCTCGTACGGCTACAGCGGCGCGGAGCTGCGGTTGCATTGGGCGC
130 140 150 160 170 180
ACCCACGACGAGACGCGAGCATGCCGATGTCGCCCGCCTCGACGCCAACGTAACCCGCG
GGCGGGGCCCCGCTCAGGATGGAGCGGCGTTCGGTACGCGCCGAGCGGCACCGGCCGGGG
190 200 210 220 230 240
CCGCCCCGGGCGGAGTCTACCTCGCCGAGCCATGCGCGGCGTTCGCCGTGGCCGGCCCC
SSII BF
GCGAAAGCGAGGAGGCAGCGAAGAGCTCCTCCTCGTCC CAGGCGGGCGCTGTT CAGGGCA
250 260 270 280 290 300
CGCTTTGCTCCTCCGTCGCTTCTCGAGGAGGAGCAGGGTCCGCCCGGACAAGTCCCGT
SSII BP
GCACGGCCAAGGTTGATTTACTCGGAACat t t t a a c t c g a t t t t t t c c c g c a t g a a a a
310 320 330 340 350 360
c g t g c c g g t t c c a a c t a a a t g a g c c t t g t a a a t a a t t g a g c t a a a a a g g g c g t a c t t t t
a g t a c t c c g t a t a a a a t t g c t a t t g g g t t t a t g c a c a g c t t g c t t g t c a a a t t c a g c a c c
370 380 390 400 410 420
t c a t g a g g c a t a t t t t a a c g a t a a c c c a a a t a c g t g t c g a a c g a a c a g t t t a a g t c g t g g
a a c t c a c c g t t t t c t g a c g g a a a t g c a g c t g c a c a t g c a g t t t t a g c c g c t c t t t t c t c a t
430 440 450 460 470 480
t t g a g t g g c a a a g a c t g c c t t t a c g t c g a c g t g t a c g t c a a a a t c g g c g a g g a a a g a g t a
g a t c a a c t a c c a c a t g t g a c a c a c g c g t g t a t a t t t t a g a t g c g g a t c g c t g a a c t c t g
490 500 510 520 530 540
c t a g t t g a t g g t g t a c a c t g t g t g c g c g a c a t a t a a a a t c t a c g c c t a g c g a c t t g a g a c
t a c t a g g t g a g c t t c g c t g a t g c t a a g t t t c t a g c t g t g t c c c a a a c c t g t t t a c t c a g g
550 560 570 580 590 600
a t g a t c c a c t c g a a g c g a c t a c g a t t c a a a g a t c g a c a c a g g g t t t g g a c a a a t g a g t c c
c t g T G G A T T C T G C T T C A C C T C C C A A T C C T T T G A C A T C T G T C T C C G A A G C A A C A G T C C A G A
610 620 630 640 650 660
G A C A C C T A A G A C G A A G T G G A G G G T T A G G A A A C T G T A G A C A G A G G C T T C G T T G T C A G G T C T
SSII BR
G C G C T T G C T T T A T G C A A T A G A C A C G C G T T T G A C T G A G T T C G T G G G G C G A G G C C A G C G G C G
670 680 690 700 710 720
C G C G A A C G A A A T A C G T T A T C T G T G C G C A A A C T G A C T C A A G C A C C C C G C T C C G G T C G C C G C

pMON810 insert sequence:

```
CAACGCCAAGCAGGACCGTCAACGTGCCCGTACTGGTTCCCTCTGGCCGCTGAGCGC
  10      20      30      40      50      60
GTTGCGGTTTCGTGCTCTGGCAGTTGCACGGGCCATGACCAAGGGAGACCGGGCGACTCGCG
      MON FOR2
CCCCAGCCCGATCGGCAAGTGTGCCACCACAGCCACCACTTCTCCTTGGACATCGATGT
  70      80      90      100     110     120
GGGGTCGGGCTAGCCGTTACACGGGTGGTGTGCGGTGGTGAAGAGGAACCTGTAGCTACA
      MONTanra
GGGCTGCACCGACCTGAACGAGGACTTTTCGGTAGCCCTTCTTTTCATTTCGGAATTTGCTTG
  130     140     150     160     170     180
CCCGACGTGGCTGGACTTGTCTCTGAAAGCCATCGGAAGAAAGTAAAGGCTTAAACGAAC
      <GM plant>
CGAGCAGTCAGGTCCTTTTGATTCATCTGAGTTTGGCTTTACAATAGCTTTTTCCTTTTCC
  190     200     210     220     230     240
GCTCGTCAGTCCAGGAAAACTAAGTAGACTCAAACCGAAATGTTATCGAAAAGGAAAAGG
      MON REV2
TTTGGCAGTACTAGTGCTTTTCATCATGAGAATCCTTCTTAGATGTAAGACCACCTGCAG
  250     260     270     280     290     300
AAACCGTCATGATCACGAAAGTAGTACTCTTAGGAAGAATCTACATTCTGGTGGACGTC
```

pOSR2A insert sequence:

```
CGAAATCGGAGTACAAAGAGGCCAACACCGTGCTAGAATCGTTACGTACCATGCAATCAT
  10      20      30      40      50      60
GCTTTAGCCTCATGTTTCTCCGGTTGTGGCAGGATCTTAGCAATGCATGGTACGTTAGTA
      OSRA-f1
CCCGACCGATCTACACTTACCATATTCCGAGAGAGAAGAAAAAGAAAACGAGGGTTGAC
  70      80      90      100     110     120
GGGCTGGCTAGATGTGAATGGTATAAGGCTCTCTCTCTTTTTTTCTTTTGGCTCCCAACTG
      OSRA-P1
ACAAAGCGAGATTGAAACCTACTCCCGAAATTCCTGTTGGACAAGGCAACAGCCACGA
  130     140     150     160     170     180
TGTTTCGCTCTAACTTTGGGATGAGGGCTTTAACGCACAACCTGTTCCGTTGTCGGTGCT
      OSRA-R1
GGACGGAGAGAGGTCGAGAGAATGTGCCATTTGTCTCAGTGGTTATGTTGTGAATGAAGA
  190     200     210     220     230     240
CCTGCTCTCTCCAGCTCTCTTACACGGTAAACAGAGTCACCAATACAACACTTACTTCT
      OSRA-R1
ATGTAGAGTGTTTCCTGTTTGCAGACAT
  250     260     270
TACATCTCACAAAGGACAAACGTCTGTA
```

pMS8 insert sequence

```
GTTGGTCGCTTAGACCATCTCCAATGTATTTCTCTATTTTTTACCTCTAAAATAAAGGAAC
  10      20      30      40      50      60
CAACCAGCGAATCTGGTAGAGGTTACATAAAGAGATAAAAAATGGAGATTTTATTTCTTG
      MS8
TCTATAATAGAGGTGGGTTTTACTCCAATGTATTTCTTTAAAATAGAGATCTCTACATAT
  70      80      90      100     110     120
AGATATTATCTCCACCCAAAATGAGGTTACATAAAGAAATTTTATCTCTAGAGATGTATA
      MS8
AGAGCAAAAATATAGAGGAATGTTATTTCTTCTCTATAAATAGAGGAGAAAATAGCAATC
  130     140     150     160     170     180
TCTCGTTTTATATCTCCTTACAATAAAGAAGGAGATATTTATCTCTCTTTTATCGTTAG
```

TCTATTTTAGAGGCCAAAAATAGAGATGGGTTGGAGTGATTTTGCCTCTAAATGCTATTAT
190 200 210 220 230 240
AGATAAAATCTCCGTTTTTATCTCTACCCAACCTCACTAAAACGGAGATTTACGATAATA

AGAGGTAGAAATAGAGGTGGGTTGGAGATGCTCTTACTATTTTCATAGTAGGTGAAAAC
250 260 270 280 290 300
TCTCCATCTTTATCTCCACCCAACCTCTACGAGAATGATAAAAAGTATCATCCACTTTTGA

TGAAACTAGAAAAGCTTCGGAGTGACGAGTGGAAAACCTCTCTTTGTAGAAACATACACA
310 320 330 340 350 360
ACTTTGATCTTTTGAAGCCTCACATGCTCACCTTTTGGAGAGAAACATCTTTGTATGTGT

TGCCATTTAGTTAACTAGTTGACATAGATTTTTGAGTCAGATAACTTTAAGAATATATAT
370 380 390 400 410 420
ACGGTAAATCAATTGATCAACTGTATCTAAAAACTCAGTCTATTGAAATTCTTATATATA

GTTTGGATGAGAGTTTGACACTTTGAGCCACTCGAAGGACAAAATTTTAAAACTTGTGGG
430 440 450 460 470 480
CAAACCTACTCTCAAACCTGTGAAACTCGGTGAGCTTCTGTTTAAAAATTTTGAACACCC

ATGCTGTGGCCATAAACCTTGAGGACGCTTTGATCATATTCTATTAACCTACAGTA **CGAA**
490 500 510 520 530 540
TACGACACCGGGTATTTGGAACCTCTGCGAAAACCTAGTATAAGATAAATTGATGTCATGCTT

MS8-F1 MS8-P1
TATGATTCGACCTTTGCAATTTTCTCTTCAGTACTCGGCCGTCGAACTCGGCCGTCGAGT
550 560 570 580 590 600
ATACTAAGCTGGAAACGTTAAAAGAGAAAGTCATGAGCCGGCAGCTTGAGCCGGCAG **CTCA**

<plant GM>
ACATGGTCGATAAGAAAAGGCAATTTGTAGATGTTAATTCCTATCTTGAAAGAAATATAG
610 620 630 640 650 660
TGTACCAGCTATTCTTTTCCGTTAAACATCTACAATTAAGGGTAGAACTTTCTTTATATC

MS8R1
TTTAAATATTTTATTGATAAAAATAACAAGTCAGGTATTATAGTCCAAGC
670 680 690 700 710
AAATTTATAAATAACTATTTTATTGTTTCAGTCCATAATATCAGGTTTCG

pRF3 insert sequence.

CGGAGGTCCGAGACGAGTTCAAAAATACATTTTACATAATATATTTTTCATATATATATA
10 20 30 40 50 60
GCCTCCAGGCTCTGCTCAAGTTTTTATGTAAAATGTATTATATAAAAAAGTATATATATAT

TATATATAACATTCAAAAGTTTGAATTATTACATAAACGTTTTCTAAATTTTCTTCACCA
70 80 90 100 110 120
ATATATATTGTAAGTTTTCAAACCTAATAATGTATTTGCAAAGATTTAAAAGAAGTGGT

AAATTTTATAAACTAAAATTTTTAAATCATGAACAAAAAGTATGAATTTGTAATATAAAT
130 140 150 160 170 180
TTTTAAATATTTGATTTTAAAAATTTAGTACTTGTTTTTCATACTTAAACATTATATTTA

ACAAAGATACAAATTTTTGATTGAAATATTGGTAGCTGTCAAAAAAGTAAATCTTAGAAT
190 200 210 220 230 240
TGTTTCTATGTTTAAAAACTAACTTTATAACCATCGACAGTTTTTTCATTTAGAATCTTA

TTAAATTAACCTATAGTAACTATATATTGAAAATATTATAAATTTTTATCAAATTCTCAT
250 260 270 280 290 300
AATTTAATTGATATCATTTGATATATAACTTTTATAATATTTAAAAATAGTTTAAAGAGTA

AAATATATAAAATAAATCTAACTCATAGCATATAAAAAAGAAGACTAATGTGGATCAAAT
310 320 330 340 350 360

TTTATATATTTTATTTAGATTGAGTATCGTATATTTTCTTCTGATTACACCTAGTTTTA
 ATTTACAGTTTTTTAGAAGTAGAATCTTTATAGTTTTATTTAAAAATATAGCAAAAATGAT
 370 380 390 400 410 420
 TAAATGTCAAAAATCTTCATCTTAGAAATATCAAAAATAAATTTTATATCGTTTTTACTA
 CACAAACCTAGCTACTTTAACCAGAAGTCCAATTCAAAAATCAAAATAAAAAATAAAAACTA
 430 440 450 460 470 480
 GTGTTTGGATCGATGAAATTGGTCTTCAGGTTAAGTTTTAGTTTTATTTTTATTTTTAGAT
 TCTAAAAAATATGTTAACTACCATGCAAAAAGTATTTTTTTTTGTAATTAGAAAACCCTGA
 490 500 510 520 530 540
 AGATTTTTTTTATACAATTGATGGTACGTTTTTCATAAAAAAAAAACATTAATCTTTGGGACT
 RF3-F1 RF3-P1
 AATTTGTACAAAATTTGGACCCCTAGGTAAATGCCTTTTTCATCTCCGGATAAGAAAAGG
 550 560 570 580 590 600
 TTAAACATGTTTTGAACCTGGGGATCCATTTACGGAAAAAGTAGAGGGCTATTCTTTTCC
 <plant GM>
 CAATTTGTAGATGTTAATTCCCATCTTGAAAGAAATATAGTTTTAAATATTTATTGATAAA
 610 620 630 640 650 660
 GTTAAACATCTACAATTAAGGGTAGAACTTTCTTTATATCAAATTTATAAATAACTATTT
 ATAACAAGTCAGGTATTATAGTCCAAGCAAAAACATAAAATTTATTGATGCAAGTTTAAAT
 670 680 690 700 710 720
 TATTGTTTCAGTCCATAAATATCAGGTTTCGTTTTTGTATTTAAATAACTACGTTCAAATTTA
 RF3-R1
 TCAGAAATATTTCAATAACTGATTATATCAGCTGGTACATTGCCGTAGAT
 730 740 750 760 770
 AGTCTTTATAAAGTTATTGACTAATATAGTCGACCATGTAACGGCATCTA