SPSD II

TRACING AND AUTHENTICATION OF GMOs AND DERIVED PRODUCTS IN THE FOOD PROCESSING AREA

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PART 1

SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

GENERAL ISSUES

AGRO-FOOD

ENERGY

TRANSPORT

.be

SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY
(SPSD II)

Part 1:
Sustainable production and consumption patterns

FINAL REPORT

Tracing and authentication of GMOs and derived products in the food-processing area

CP/32

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1 INTRODUCTION

1.1 CONTEXT

Genetically modified organisms (GMOs) are living organisms with engineered genetic modification of their genome. The additional exogenous gene(s) express a protein responsible for new agronomic trait(s) of economical interest.
In the European Union (EU), acceptance of GMOs entering the food/feed chains has to face an increasing opposition of consumers and their respective stakeholders. The regulations 258/97(1), 1139/98 (2) and 49/2000 (3) meet the consumers and authorities concerns as they require the labelling of food and/or derived products containing more than 1% of GM material. The directive 2001/18/EC (4) enlarged the labelling requirement to the feed products and set the bases for traceability of GMOs. With the new regulations on ‘GMO food and feed’ 1829/2003/EC (5) and ‘traceability and labelling’ 1830/2003/EC (6), the EU exempts thresholds for the mandatory labelling of the unavoidable, adventitious presence of transgenes in marketed food and feed products. The threshold for the adventitious presence of GM material in food/feed products has been set to 0,9%. Commission Recommendation 2004/787/EC (7) on ‘technical guidance for sampling and detection of GMOs’, set up as a helpful tool for practical implementation of Regulation 1830/2003, states that ‘the results of quantitative analysis should be expressed as the number of target DNA sequences per target taxon-specific sequences calculated in terms of haploid genomes’. This new accepted ‘haploid genomes-unit’ for measurement and, more importantly, also for expression of a GMO content, solves a lot of problems and makes a horizontal implementation of the legislation feasible. This new definition should be used along the food and feed chain and should also be reflected in the seeds regulation, which is currently under development.

1.2 OBJECTIVES

To allow the Members States to apply the regulatory requirements, it is necessary to supply them with analytical detection tools for authentication and quantitation of these authorized GMOs. The present project, representing a joint research work of five different teams, aimed at providing Belgium with several up to date technologies allowing tracing, authentication and quantitation of GMOs and derived unprocessed and processed ingredients in the food-processing area.
Development of these technologies, namely qualitative/quantitative Polymerase Chain Reaction (PCR)-based methods and GMO biochip, are built on the existence of specific GMOs genetic markers. Among these markers, some are generic to different GMOs (expression control sequences such as promoter 35S, terminator Nos,...), some are event-specific (junctiun sequences between the transgene and the plant insertion locus). Set up of analytical methods targeting such sequences relies on the availability of certified reference material (CRM) to be used as negative/positive controls. The search for GM-crops CRM highlighted the difficulty to get reference material for authorized or non-authorized GM-crops (production, availability, cost,..).
As an alternative to this first issue, ISSP-WIV (Partner #1) has proposed to SSTC-DWTC to develop first with the CLO (partner #2), a bank of plasmid-cloned analytes as reference material (task A.1 under theme 1 of the project). In the present case, the analytes are species-specific or GM -specific genetic markers (DNA sequences). These plasmids with cloned

genetic markers have to be calibrated in regard of GM-crops certified reference material (CRM) for their use as standard material both for quantitative and qualitative PCR (task common to partners #1, #2 and #5). One should be aware of the increasing number of GMOs that should enter the European market in the coming years. Use of an automated analytical method allowing the simultaneous detection of different GMOs in a single round will make possible a broad screening of GMOs at low cost. In this context, the development of qualitative Biochip has been set as another challenge of this project (theme 2 of the project). This technique relies on hybridization of PCR products with specific GMOs probes fixed of on a solid matrix. The FUNDP (partner #3) has to develop the prototype of this qualitative biochip while ISSP-WIV should perform the validation.
Finally, quality of extracted DNA is a critical factor for the successful development DNA based analytical detection methods. Theme 3 of this project is to analyse, describe and rationale the link between the quality /purity of a DNA solution and the quantitation of GMOs analysed by the mean of real time PCR (Partners #4 and #5) and consequently establish a quantitative acceptance criteria for DNA extraction methods.

2 STATE OF THE WORK OF THE PROJECT

2.1 THEME1: PLASMID AS GMOS REFERENCE MATERIAL (PARTNERS #1, #2 AND #5)

The availability of certified reference materials (CRMs) and calibrators as control materials is of major concern in the field of GMO analysis. Reference materials (RMs) are used for calibration and traceability assessment, method validation, statistical quality control (QC) and to provide proof of the laboratories competence [8,9]. For GMO analysis, the choice of RMs has been debated among experts in the EU for years by now. The main question in this discussion was whether RMs and calibrators should be matrix-based or pure DNA analytes.
The haploid genomes unit requires that RMs and calibrators allow to quantify and calculate a GMO content in terms of haploid genomes. Therefore, pure DNA analytes are a better choice than matrix-based RMs. Advantages of pure DNA RMs are the universal applicability and cheap and fast production and distribution possibilities. Analyte RMs are only applicable for the analyte determination step as such, i.e. the PCR, and do not apply for the whole GMO analysis procedure. This does not present a problem, to the contrary, is in line with the ‘modular validation approach’, which has been accepted in the EU. Modularity refers to the different steps or modules in the GMO detection approach, which need to be considered independently and separately from each other [10]. As such, the distinguished steps – sampling, sample preparation, DNA extraction, and PCR – need to be validated separately, and require RMs and calibrators which are representative for the input in and fit for the particular purpose of each analytical step. For the PCR step, this means that pure DNA solutions could be used as calibration standards, matching as much as possible the sample, which is being analyzed.
As pure analyte standards, there is a choice between genomic and plasmid DNA. Genomic DNA needs to be extracted from a matrix first and thus is liable to matrix effects and processing influences such as degradation. Plasmid DNA vectors, containing the sequence(s) of interest, are easier to handle because of their simple and cheap production process, their stability as well as universality and wide applicability.

In this context, we aimed at developing plasmid DNA markers allowing the qualitative detection and the quantitation of different GMOs, noteworthy those authorized in EU. The use of these plasmid as calibrators molecule should be demonstrated through the development of real-time PCR assays included (1) the design and testing of a set of primers and a fluorescently labelled TaqMan probe, (2) the optimization of PCR conditions for each of the primer/probe sets, and (3) the combination of a GM event-specific PCR assay with an endogenous reference gene specific assay, in order to be able to quantify the relative GMO content (% genomes/genomes). Testing of the new markers as calibrators in those PCR methods, included a comparative study between several types of DNA standards (tasks common to partners #1, #2 and #5).

Another objective was to investigate the influence of the genetic structure of seeds on real-time PCR quantitative results obtained for seed lots. The specific composition of a seed kernel, the way of inheritance and the ploidy level make that there exists a discordance between a GMO % expressed as genomes and a GMO % based on numbers of seeds. This means that a threshold fixed as a percentage of seeds cannot be used as such for real-time PCR. We studied the extent of those influences and the consequences if real-time PCR is used for quantification of GM seeds (partners #2 and #5).

2.1.1 The ENGL plasmids

2.1.1.1 Construction of plasmids DNA markers for GMOs

One goal of this project was to develop plasmids containing GMOs genetic markers to be used as RMs. These single target plasmids (STP) are either GMO event/construct-specific or endogenous species/cultivar-specific. The event specific target consists of junction regions or other sequences resulting from the recombination process between the transgene DNA and the host plant DNA, which can be regarded as unique for a specific transgene event. Junction regions or edge fragments are the DNA sequences at the transition between the plant DNA and the inserted T-DNA. This implies that the event specific constructed plasmids are unique identification markers. The map of different targeted event-specific sequences are represented in Figure 1.

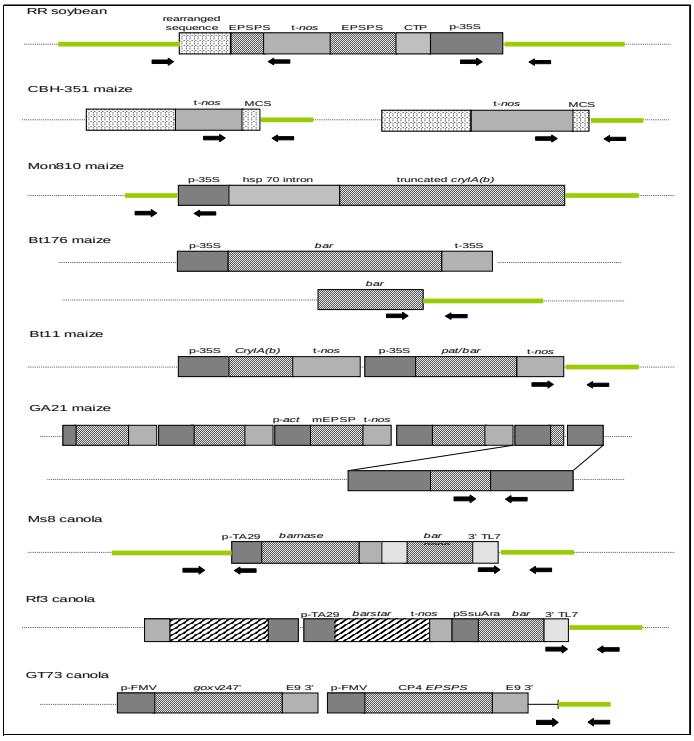


Figure 1 Schematic presentation of the transgenic maps of different events used in this study. The arrows indicate the position of primers developed by partner#2 to clone junction sequences from this event.

The first year of the project settled the bases for the construction of a bank of cloned GMO markers. 18 plasmids with cloned GMO-specific and endogenous species-specific markers were constructed. The setup for the construction, the development and storage of these plasmids was harmonized for all partners involved in the project. It was also decided to build a standard scientific dossier associated with each plasmid. This dossier contains all relevant information about the host organism, the plasmid vector and the inserted sequence. More in particular, the bacterial growth conditions, the complete DNA sequence of the plasmid including the cloned sequence, the protocol used for construction of the plasmid, details about the cloned sequence including EMBL or GenBank accession numbers and relevant literature references are also indicated.
Moreover, it was agreed that the plasmids would be encoded as pENGL-xx-yyy’. “ENGL” refers to the approval of this bank by the European Network of GMOs Laboratories (ENGL) on December 5th 2002. 'xx' refers to an assigned code for each partner involved in the project, while 'yyy' is a code given by the laboratory that constructed the plasmid. The official pENGL code is given once a constructed plasmid has been given to the ISP/WIV (Partner #1) who centralize and coordinate the maintenance of pENGL. The pENGL have to be then deposited in the official plasmid and cDNA collection (LMBP) from the Belgian Coordinated Collection of Micro-organisms (BCCM^{TM}) (task A.2 under theme 1) of Gent as central master depository under the statute of full private deposit. BCCM^{TM} is a consortium of four complementary research-based culture collections, coordinated by the Belgian Office for Scientific, Technical and Cultural Affairs (OSTC). BCCM^{TM} entails a plasmid and cDNA collection (LMBP), a bacteria collection (LMG), a collection of biomedical fungi and yeasts (IHEM) and an (agro)industrial fungi and yeasts collection (MUCL). The BCCM^{TM}/LMBP collection, centralized at Ghent University, contains more than 1500 plasmids and circular double-stranded DNA molecules. For each public deposit, both a plasmid DNA solution and a stab-culture of the *E. coli* host organisms were asked, together with a dossier containing molecular details about the host organism, the plasmid vector and the inserted sequence. More in particular, the bacterial growth conditions, the complete DNA sequence of the plasmid including the cloned sequence, the protocol used for construction of the plasmid, details about the cloned sequence including EMBL or GenBank accession numbers and relevant literature references were required.
Along the second year, we further developed first generation plasmids containing one genetic marker. Also promoter or terminator sequences are exploited as markers for mass screening of GM-crop. Second generation ENGL plasmids with two genetic markers were also constructed. These so-called "pJANUS plasmids" contain both the GM transgenic target sequence and the species/cultivar-specific sequence. These plasmids are ideal tools for quantitative PCR since the co-cloning of both sequences ensures a perfect 1:1 ratio over all the points of a standard curve in RT-PCR.

The initial collaboration between ISP-WIV (partner #1) –and CLO (partner #2) for the construction of a plasmid bank has been enlarged to other non-financed partners: The CRA, the INRA, the IHCP (Ispra), the IRMM (Geel) and the DGCCRF (Strasbourg). A common coding system was agreed and applied. For 1st step and final step cloning stages.
At the end of the present project, the number of constructed plasmid has been raised to 151. Among these plasmids, 56 are in the denominated as pENGL plasmids, allowing their deposit to BCCM. Table 1-4 summarize the list of the constructed by the different partners.

	Gene Trait sequence	Plasmid name	Characteristics of gene trait	Insert size	Bank Access Number	Seq. Position
species	cruA	pENGL-02-012	oilseed rape (Brassica napus)	394bp	X59294.1	790-1183
	PEP3-PEPcase	p02-020		1005bp	D13987	2652-3637
		p02-021		1288bp	D13987	2601-3889
	zein	p02-016	maize (Zea mays L.)	531bp	U25674	1-531
		pENGL-02-017		277bp	M23537.1	1194-1470
	Le1	p02-018	soybean (Glycine max L.)	622bp	K00821	1114-1736
		pENGL-02-019		178bp	K00821	1559-1736
		pENGL-02-001		948bp	K00821	934-1881
	PEP3-PEPcase	p02-020	oilseed rape (Brassica napus)	1005bp	D13987	2652-3637
		p02-021		1288bp	D13987	2601-3889
	tRNA-Leu	pENGL-02-022	Maize gene for tRNA-Leu	533bp	ZMA 86563	48976-49508
		pENGL-02-023		122bp	ZMA 86563	49387-49508
screening	CaMV ORF V, CRT	pENGL-02-002	CRT from Cauliflower mosaic virus	191bp	M90542	3804-3993
	CaMV ORF VI	pENGL-02-003	inclusion body matrix protein from Cauliflower Mosaic virus	383 bp	V00141.1	6328-6710
	p35S	pENGL-02-013	35S promoter from Cauliflower Mosaic Virus	123bp	V00141	7313-7435
		pENGL-02-014		147bp	V00141	7249-7395
	tNOS	pENGL-02-015	Nos terminator from *Agrobacterium tumefaciens*	118bp	V00087	1955-2082
	t35S	p02-026	35S terminator from Cauliflower Mosaic Virus	119 bp	V00141.1	7481-7630
event	CBH-351 (Starlink)	pENGL-02-009	CBH-351 plant DNA & tNOS	178bp	confidential	confidential
	MS 8	pENGL-02-010	junction plant & vector	280bp	confidential	confidential
	Rf 3	pENGL-02-011	junction plant & vector	215bp	confidential	confidential
	T 25	p02-025	p35S & T 25 plant DNA	152 bp	confidential	confidential
	Bt 11(*)	pENGL-05-004	plant DNA Bt 11 & junction	207 bp	Publication	Publication
construct	p35S & cry9C(*)	pENGL-05-003	from CBH 351	379	confidential	confidential
	p35S & pat	pENGL-02-024	from Topas 19/2	321 bp	confidential	confidential
	pat	p02-004	gene encoding phosphinotricin herbicide tolerance (glufosinate)	550bp	Home	1-550
	cry9c	p02-005	gene encoding insect resistance to European corn borer (ECB) (CBH-351, Starlink)	924bp	Home	18-941
		p02-006		1019bp	Home	511-1530
		p02-007		918bp	Home	960-1878
		pENGL-02-008		180bp	A73544	1063-1243

Table1: overview of plasmids made by ISP-WIV (partner #1, ENGL code 02): CRA (partner #5, ENGL code 05) has cloned two fragments of interest into the intermediate plasmid while sub cloning in pUC18 was performed by ISP-WIV

	Gene Trait sequence	Plasmid name	Characteristics of gene trait	Insert size	Bank Access Number	Seq. Position
species	zein	pENGL-03-003	endogenous gene from maize (Zea mays L.)	485 bp	M23537	1151-1635
	zein	pENGL-03-008		70 bp	M23537	1317-1386
	IVR	pENGL-03-009		105 bp	U16123	2289-2393
	IVR	pENGL-03-018			U16123	
	adh	pENGL-03-010		138 bp	K03285	70-207
	hmgA	pENGL-03-011		79 bp	AJ131373	719-797
	zein & IVR	pENGL-03-016	endogenous gene from maize (Zea mays L.)	175 bp	M23537 & U16123	1317-1386 & 2289-2393
	adh & hmgA	pENGL-03-017		217 bp	K03285 & AJ131373	70-207 & 719-797
	zein & IVR & adh & hmgA	pENGL-03-012		392 bp	M23537 & U16123 & K03285 &	1317-1386 & 2289-2393 & 70-207 & 719-797
screen	tNOS	pENGL-03-001	Nos terminator from *Agrobacterium tumefaciens*	199 bp	AE009420	2688-2886
	p35S	pENGL-03-002	35S promoter from Cauliflower Mosaic Virus	244 bp	NC 001497	7192-7435
construct	Cry1Ab	pENGL-03-004	gene encoding insect resistance to European corn borer	1914 bp	Home	Home
JANUS	pat & zein	pENGL-03-006		664 bp	home & M23537	home & 1151-1635
	p35S & zein	pENGL-03-007		729 bp	X79465 & M23537	7192-7435 & 1151-1635

Table 2: Overview of plasmids received from IHCP and registered at the WIV-SBB

	Gene Trait sequence	Plasmid name	Characteristics of gene trait	Insert size	Bank Access Number	Seq. Position
event	Bt 11	p01-018	tNOS & Bt 11 plant DNA	505 bp	Home	Home
	Bt 176	p01-017	event-specific	570 bp	Home	Home
		p07-016	junction plant & vector	150 bp	Home	Home
	GT 73	p01-013	junction plant & vector	512 bp	Home	Home
	GTS 40-3-2	pENGL-01-001	GTS 40-3-2 plant DNA & pe35S	359bp	AJ308514	1-359
		pENGL-01-003	tNOS & GTS 40-3-2 plant DNA	928bp	Home	confidential
		p01-012	pe35S & GTS 40-3-2 plant DNA	422bp	Home	Home
		p03-014	GTS 40-3-2 plant DNA & pe35S & CTP4 & CP4 EPSPS	775 bp	home	home
		p07-021	GTS 40-3-2 plant DNA & pe35S	359 bp	AJ308514	1-359 bp
	HCN 10, HCN 20	p07-009	junction plant & vector	192 bp	Home	Home
	Mon 810	p01-009	Mon 810 plant DNA & pe35S	901bp	AF 434709	1-901
		pENGL-04-0012	Mon 810 plant DNA & pe35S	186bp	Home	confidential
		p07-006	junction plant & vector	380 bp	Home	Home
		p07-011	junction plant & vector	90 bp	Home	Home
	MS 8	p01-006	MS 8 plant DNA & pTA29	792bp	Home	Home
		p01-010	tg7 & MS 8 plant DNA	629bp	Home	Home
	RF 3	p01-011	tg7 & RF 3 plant DNA	513bp	Home	Home
	T 25	p07-017	junction plant & vector	107 bp	Home	Home
construct	ORF III CaMV	p07-010	ORF III from Cauliflower Mosaic Virus (CaMV)	260bp	Home	Home
		p07-013		260bp	Home	Home
		p07-014		260bp	Home	Home
		p07-015		260bp	Home	Home
	pat	p03-005	gene encoding phosphinotricin herbicide tolerance (glufosinate)	179 bp	home	home
	cry1Ab	p04-0021	gene encoding insect resistance to European corn borer	129bp	E29366	confidential
		p07-004		211bp	Home	Home
	mEPSPS	p07-018	gene encoding glyphosate tolerance	1000 bp	Home	Home
		p07-019		700 bp	Home	Home
	p35S & IVS 2 & pat	pENGL-04-0008	from Bt 11	700bp	Home	confidential
	IVS 6 & Cry1Ab & tNOS	p04-0005	from Bt 11	2.1 kbp	publication	publication
	p35S & IVS 6 & Cry1Ab	p04-0006	from Bt 11	1.2 kb	publication	publication
	IVS 2 & Pat	p07-006	from Bt 11	189 bp	Home	Home
	p35S & IVS 2 & pat	p07-020	from Bt 11	1000 bp	Home	Home
	pCDPK & Cry1Ab	pENGL-04-0015	from Bt 176	227bp	Home	confidential
	pPEPC & Cry1Ab	pENGL-04-0017	from Bt 176	948bp	Home	confidential
	pCDPK & Cry1Ab	p04-0023	from Bt 176	900bp	publication	publication
	Cry1Ab -PEPC intron 9 & p35S	p04-0019	from Bt 176	2.1 kb	publication	publication
	p35S & bar	p04-0020	from Bt 176	600bp	publication	publication
	first insert CBH-351 & tNOS	p01-007	from CBH-351 (Starlink)	549bp	Home	Home
	second insert CBH-351 & tNOS	p01-008	from CBH-351 (Starlink)	887bp	Home	Home
	pe35S & CTP4	pENGL-05-001	from GTS 40-3-2	172bp	Home	Home
	pe35S & hsp70 & Cry1Ab	p04-0004	from MON 810	1.4 kbp	publication	publication
	pe35S & hsp70 & Cry1Ab	p07-031	from MON 810	1600 bp	Home	Home
	p35S & Pat & t35S	p07-008	fromT 25	1100 bp	Home	Home
	p35S & pat & t35S	p07-027	from T 45	800 bp	Home	Home
	p35S & pat & t35S	p07-028	from Topas 19/2	800 bp	Home	Home
	p35S & pat & t35S	p07-033	from T 25	800 bp	Home	Home
	p35S & t35S	p07-012	from Bt 176	170 bp	Home	Home
	p35S & t35S	p07-012	from Bt 176	170 bp	Home	Home

Table 3: Overview of event-specific s and construct-specific plasmid planned or already constructed but not yet registered at the WIV-SBB

	Gene Trait sequence	Plasmid name	Characteristics of gene trait	Insert size	Bank Access Number	Seq. Position
species	cruA	p01-ND5	oilseed rape (Brassica napus)	+/- 700bp	Home	Home
	zein	p01-015	maize (Zea mays L.)	655bp	Home	Home
	IVR	p07-003		179bp	Home	Home
		p01-ND3		not yet defined	Home	Home
		p01-ND4		+/- 700bp	Home	Home
	adh	p07-005		134bp	Home	Home
	hmgA	p04-0022		95bp	AJ131373	719-797
	zSSIIb	p04-0016		155bp	AF019297	349-504
		p04-0024		235bp	AF019297	329-563
	Le1	p01-014	soybean (Glycine max L.)	795 bp	Home	Home
		p03-013		414 bp	K00821	1099-1512
		pENGL-05-002		171bp	K00821	1183-1253
		pENGL-01-002		118bp	K00821	1215-1332
	SBSPS	p07-022	sugar beet	1600 bp	X81975	Home
		p07-023		1600 bp	X81975	Home
		p07-024		1600 bp	X81975	Home
		p07-025		800 bp	X81975	Home
		p07-026		1600 bp	X81975	Home
screen	p35S	p07-001	35S promoter from Cauliflower Mosaic Virus	246bp	Home	Home
		p07-002		266bp	Home	Home
PLANTS	GTS 40-3-2 plant DNA & Le1 & pat	p03-015	multitarget fragment	1368 bp	Home & K00821 & Home	Home & 1099-1512 & home
other	preinsertion (promotor locus)	pENGL-01-004	preinsertion (35S) loci soybean for GTS 40-3-2	470bp	Home	confidential
	preinsertion (terminator locus)	pENGL-01-005	preinsertion (tNOS) loci soybean for GTS 40-3-2	773bp	Home	confidential

Table 4: Overview of species-specific, screening plasmids planned or already constructed but not yet registered at the WIV-SBB

2.1.1.2 The ENGL plasmid database

The follow up of the different constructed plasmids requires the ability to manage all informations relative to this particular RMs. For this purpose, the ISP (partner #1) initiate the construction of an official pENGL database listing the different cloned genetic markers (Figure 2). The database links the molecular data of GMO events placed on the world market to the plasmids containing genetic markers for detection and quantification of these GMO events and to the related PCR primers. The molecular data of these GMO events was collected from regulatory dossiers, the BATS report and the Agbios database. The SSTC partners will have access to the full database including plasmids under construction.
Once the plasmids will be deposited in the BCCM collection, the ENGL plasmids will become public available. At that moment, a public database containing all necessary information of the ENGL plasmids should be accessible on the web. This public database was constructed based on the FileMaker database mentioned above, containing the same easy-to-use search tool. As shown in Figure 2 the user can search for a specific GMO event or a specific genetic marker.

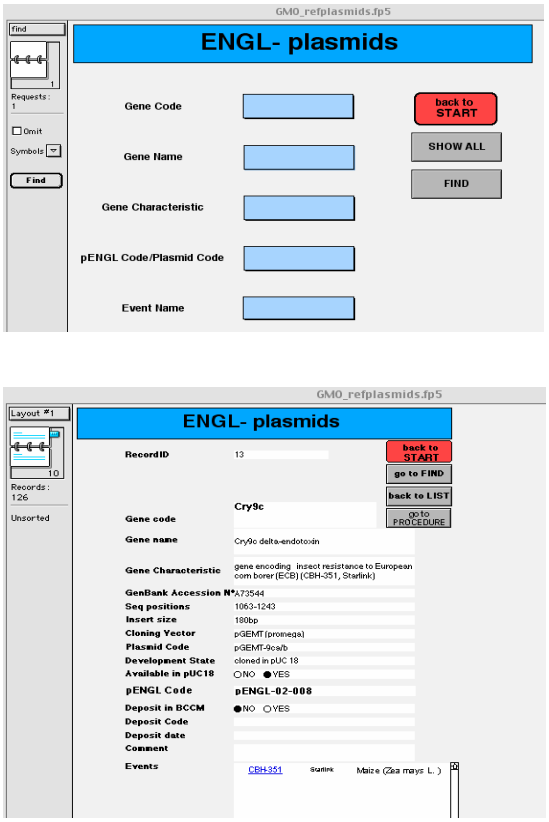


Figure 2: ENGL plasmid database in FileMaker

2.1.1.3 Discussion and conclusions

The production process of the plasmids is a simple, straightforward, two-steps cloning process, consisting of a ligation and a tranformation. Not only are they easily produced and stored, a major strength of plasmid DNA standards is found also in their universal applicability. Whichever DNA fragment can be cloned, from taxon-specific gene segments to event-specific border sequences. The target-specificity of the plasmids makes them very useful as positive control samples or as calibrators in target-specific PCR reactions. Oppositors of plasmid DNA claim that this point exactly is the main drawback: that plasmid DNA solutions have a limited applicability as they can only be used for one single PCR. However, target-specific, pure DNA RMs is nowadays needed for implementation of the modular GMO analysis procedure.

Insight in this modularity of the analytical procedure for GMO analysis and of validation approaches has formed the basis for this work. It is clear that matrix RMs, containing a certain mass amount of GM material in a matrix of non-GM material, and from which genomic DNA could be extracted, were not suitable for quantification of a DNA sequence, isolated from a completely other matrix material. Matrix RMs are limited in use as they only apply to exactly the same matrix. Moreover, the CRMs for GMOs, commercially available from the end of the nineties, have known problems with protein and DNA degradation and thus with the accuracy of the certified values.

The introduction of plasmid DNA diagnostics as a new type of RMs for GMO analysis is a

big step forwards in the standardization of methods for GMO analysis. Up till now, harmonization of analytical methodologies was hampered to a great extent by the lack of suitable RMs. CRMs for GMOs were available only for a limited number of transgene events, in a limited range (0-5 %) and in one particular matrix material. Over more, the cost price for these CRMs is very high, due to the expensive and cumbersome production and certification process of matrix RMs. Plasmid calibration standards already exist for a high number of commercialized GMOs and in a wide range for quantification (1-10^6 copies). Although they are non-certified, our plasmids can be considered as RMs, usable as positive control samples in qualitative GMO testing, and as calibrator for accurate quantification of GMOs

2.1.2 Validation of pENGL use for qualitative purposes

2.1.2.1 The design of a GMO screening plate

We assess to evaluate the use of single target sequence plasmid in qualitative analyses. We develop to this end a semi-quantitative screening plate using the SYBRgreen technology.
The fluorescent dye SYBR Green I binds to the minor groove of the DNA double helix. In solution, the unbound dye exhibits very little fluorescence, however, fluorescence is greatly enhanced upon DNA-binding. During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls. Fluorescence measurement at the end of the elongation step of every PCR cycle is performed to monitor the increasing amount of amplified DNA. Together with a melting curve analysis performed subsequently to the PCR, the SYBR Green I format provides an excellent tool for specific product identification and, if standardized, for quantification.
The overall aim in our context is to develop a general “easy-to-use” GMO screening plate for semi-quantitative detection of GMOs in food samples using SYBRgreen and plasmid RMs as controls.
The preliminary tests have been realized as followed. First, the PCR reactions used in the ISP GMO laboratory (Partner #1) according to the ISO17025 norms were performed using the ABI7700 thermocycler and the SYBRgreen mastermix. The reactions were carried out with certified reference materials from Fluka (IRMM-Geel). The results obtained on the ABI7700 using the SYBRgreen technology (Ct values and melting curves) comply with those obtained by the qualitative PCR visualized on agarose gel.
In the next step, the PCR reactions (on the ABI7700 with SYBRgreen) were carried out using ENGL plasmids as positive controls. These ENGL plasmids comprise the corresponding PCR fragments as genetic markers. For each plasmid the specificity, the limit of detection (LOD) and the repetability of the LOD were determined. An example for the specificity is provided in figure 3.

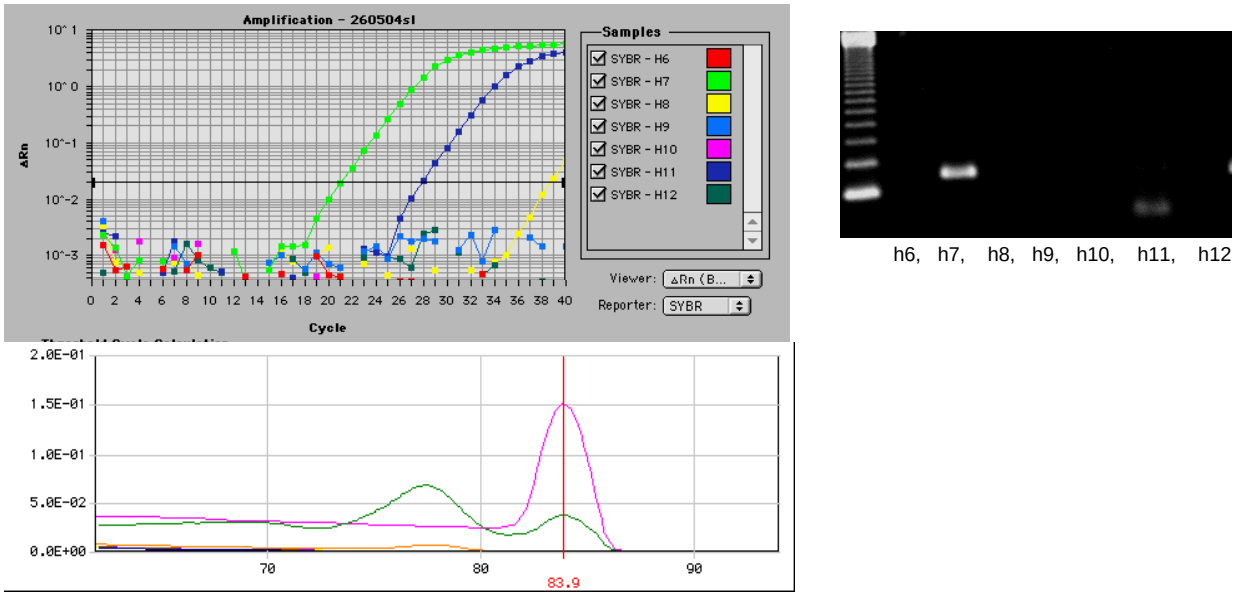


Figure 3: specificity of the SL9/SL10 primers for the lectine amplicon for different pENGL. The ENGL-02-023 plasmid (h6) contains the fragment corresponding the PL1/PL2 PCR. Similarly, the ENGL-02-019 (h7) plasmid contains the fragment of the SL9/SL10 PCR, the ENGL-03-010 plasmid (h8) the fragment of the ADH-1 F/S PCR, the ENGL-02-014 plasmid (h9) the fragment of the 35S 3/6 PCR, the ENGL-02-015 (h10) plasmid the fragment of the HA-NOS 118 F/R PCR and the ENGL-02-002 (h11) plasmid the fragment of the CRT F/R PCR. h12 correspond to the No Template Control.
When analysing a sample both the Ct (threshold cycle), the results of this experiment demonstrate that only the ENGL-02-019 (h7) plasmid containing the fragment obtained following the SL9/SL10 PCR is positive. It shows an amplification curve with a Ct (= 21), which is significantly lower than the Ct of the no template control (Ct = 40) or the other plasmids (Ct between 38 and 40). Secondly, the ENGL-02-019 plasmid is characterised by a specific melting point of approximately 84°C. The ENGL-02-002 (h11) plasmid containing the CRT fragment displays a low Ct (=27), however this plasmid is characterized by a non-specific, low melting point suggesting primer-dimer formation. The results obtained on gel confirmed the results of the SYBRGreen PCR.

An in house validation of the screening plate has successfully been performed for a set of 6 plasmids containing the screening markers used in routine analysis in the GMOlab. In the future, the screening plate should be produced and validated in collaboration with Diagenode and the European network of GMO laboratories (ENGL), respectively. Further, it is planned to extend the screening plate with an identification plate for those GMOs allowed on the European market.

2.1.2.2 **Validation of a pENGL mix**

We also investigate use of a plasmid mix as standard for multiple qualitative routine analyses. In the course of the present work, five plasmids each containing a single amplicon were mixed. These amplicons are the one amplified in the lab for routine analysis under ISO17025 norms. When compared to the amplification of a single plasmid, the plasmid mix gives in the same experimental procedure the expected amplicon. This demonstrates that multiple plasmids with different single markers could be the amplified from a mix.

2.1.3 Plasmid calibrants for quantification of GMOs using real time PCR

So far, the determination of GMO content is estimated by the mean of real-time PCR. The level of GM is calculated by the ratio of the transgene copy number to an endogen copy number. This relative quantition is able by the use of standard curves established with certified reference material (CRM). As already mentioned, the production of CRM is time consuming and further, CRMs are not available for all genetically modified events in Europe.
The plasmids containing GMOs insert are planned to be used as calibrant noteworthy for quantitative PCR. Double targets plasmids (endogene in cis with the transgene) are good candidate to replace the CRM as pure calibrator molecules for the relative quantitation of traces of GMOs in food/feedstuffs.

2.1.3.1 Preparation of plasmid DNA calibrators

For setting up real-time PCR calibration curves and developing absolute and relative quantification methods, both CRMs and plasmids containing GMOs markers were used.
For plasmids, primary solutions were prepared from bacterial cultures by means of Plasmid Prep Kit. Plasmid DNA concentrations were determined. Taking into account the molecular weight of double stranded DNA (965 Mb weigh 1 pg) [11] and the size of the plasmid, the number of copies of the inserted target sequence was calculated. The primary stock solution was first diluted to a primary working solution of e.g. 10^8 cp/5 µl, from which tenfold dilutions were made from 10^7 or 10^6 copies (highest concentration used in real-time PCR) to 10 copies or one single copy per reaction tube (lowest concentration used in real-time PCR). Those tenfold dilutions were used as calibrator series in all real-time PCR reactions.

2.1.3.2 Design of event-specific primers and probes

If not described in the literature, primers were designed based on the sequences of the cloned DNA fragments These primers should allow amplifying ideally a fragment of 400 to 500 base pairs. We put forward that the same primer sets should be used for both qualitative and quantitative PCRs. For the quantitative assays, a TaqMan probe was in addition designed, annealing between the forward and the reverse primer. In addition, the following rules for probe design are foreseen (1) no G at 5' (to avoid quenching of the reporter dye), (2) more C's than G's (because of quenching activity of G bases), (3) not more than two C's or G's in the last 5 bases at the 3'-end and (4) if possible, not more than three equal, sequential bases in the whole probe. The probes are labelled at the 5' end with a fluorochrom, the main in use being the reporter dye FAM. For eventual duplex amplification of both the event-specific target and the endogenous target, the second probe could be labelled with another dye. In this way, the group of CLO (partner #2) assays the real time PCR with 5' FAM labelled probe for the event-specific amplicon and a 5' JOE labelled probe for the endogenous amplicon. All probes are labelled with the quencher dye TAMRA at the 3'-end.
The names and sequences of all used oligonucleotides by CLO (partner #2) are shown in **Table 5**, together with the plasmid clones to which they are applicable. Table 5 shows that the primer/probe set for the soybean-specific lectin (*Le1*) gene can be applied for two different plasmid markers each. In a similar way, more than one plasmid marker is available for quantification of the RRS event, based on either the p-35S- or the t-*nos*-border. In other words, different plasmids can be chosen as calibrators in the real-time PCRs for those targets.

Event	Sequence	Oligo	Orientation	Sequence	Amplicon (bp)	Name of plasmid
soybean	lectin gene	LEC-1	forward	5'-CATCCACATTTGGGACAAAG-3'	96	pAS106 pCM9
		LEC-2	reverse	5'-TCTGCAAGCCTTTTTGTGTC-3'		
		TQ-LEC-2	forward	5'-JOE-CTTCGCCGCTTCCTTCAACTTCAC-TAMRA-3'		
maize	zein gene	ENDOZEIN-1	forward	5'-GCATTGTTCGCTCTCCTAGC-3'	110	pCM10
		ENDOZEIN-2	reverse	5'-TACTGCATGCATGGGTTCAT-3'		
		TQ-ZEIN	forward	5'-JOE-TTCCAGGGCACTTGCCACCA-TAMRA-3'		
canola	cruciferinA gene	CRUKZ-1	forward	5'-TGTTAGGACAGCGCAACAAC-3'	121	pCM11
		CRUKZ-2	reverse	5'-AACTTCCTCCTGCGGTCTCT-3'		
		TA-CrucA-2	forward	5'-JOE-TCCGAGTCCAAGGCCCATTCAGT-TAMRA-3'		
RR soybean	p-35S border	35S3	forward	5'-CATCTTTGGGACCACTGTCG-3'	123	pAS104 pCM7
		RRS5	reverse	5'-ACAGGTTAAAATAAACATAGGGAACC-3'		
		TQ-35S junc	forward	5'-FAM-AACGATGGCCTTTCCTTTATCGCAAT-TAMRA-3'		
	t-*nos* border	nos-junc-F1	forward	5'-CCCTAATAGGCAACAGCATGA-3'	189	pAM1
		primer E	reverse	5'-GAGAACTACCTTCTCACCGCAT-3'		
		TQ-NOS junc	forward	5'-FAM-CGCGGCCATCGTCGAGAAGTTC-TAMRA-3'		
CBH-351 maize	t-*nos* border B	NOS RR1	forward	5'-AGCGCGCAAACTAGGATAAA-3'	111	pCM3
		SL junc3	reverse	5'-CGTTCTGGGAAGGATAGAATCGTC-3'		
		TQ-SLnosB	forward	5'-FAM-CGCGCGCGGTGTCATCTATG-TAMRA-3'		
MON 810 maize	p-35S border	MON810-plant-F2	forward	5'-ATAACCTTCGCCCGAAAATC-3'	180	pCM4
		Mon810-35S-R1	reverse	5'-CAACGATGGCCTTTCCTTTA-3'		
		TQ-Mon810	forward	5'-FAM-CATCCTTTGCCATTGCCCAGC-TAMRA-3'		
Bt176 maize	*bar* gene border	Bt176-b	forward	5'-GTGCATCAATGGAGGAGAGAAC-3'	77	pCM12
		Bt176-a	reverse	5'-GACTTCAGCCTGCCGGTACT-3'		
		TQ-Bt176	forward	5'-FAM-TCTCGGTGACGGGCAGGACC-TAMRA-3'		
Bt11 maize	t-*nos* border	Bt11-a	forward	5'-GCGGAACCCCTATTTGTTTA-3'	93	pCM13
		Bt11-b	reverse	5'-CAAGAAATGGTCTCCACCAAA-3'		
		TQ-Bt11	forward	5'-FAM-TATCCGCTCATGGAGGGATTCTTGGA-TAMRA-3'		
GA21 maize	internal segment	GA21-a	forward	5'-AGAGCTGTAGTTGTTGGCTGTG-3'	88	pCM14
		GA21-b	reverse	5'-GCTGGGGGATCCACTAGTTCT-3'		
		TQ-GA21	forward	5'-FAM-TGGAAAGTTCCCAGTTGAGGATGCT-TAMRA-3'		
Ms8 canola	p-TA29 border	Ms8-LB3	forward	5'-AGCCGGCTATTTGTGTAAAA-3'	110	pCM1
		Ms8-LB4	reverse	5'-AAAAAGGAGGGTGTTTTTGG-3'		
		TQ-Ms8LB	forward	5'-FAM-CGACGGATCCCCGGGAATTC-TAMRA-3'		
Rf3 canola	3' TL gene 7 border	Rf3-RB3	forward	5'-GGAATTAACATCTACAAATTGCCTTT-3'	100	pCM6
		Rf3-RB8	reverse	5'-TTGTAATTAGAAACCCTGAAATTTGT-3'		
		TQ-Rf3RB	forward	5'-FAM-TCGCGAGATGAAAAAGGCATTTACC-TAMRA-3'		
GT73 canola	3' border	GT73-b	forward	5'-TAGATTTCCCGGACATGAAGAT-3'	106	pCM8
		GT73-a	reverse	5'-TCAGCAAGATTCTCTGTCAACAA-3'		
		TQ-GT73	forward	5'-FAM-TCCTTTTCTTGCCTTCGTATAAGCTTGTG-TAMRA-3'		

Table 5: Primers and TaqMan probes used in qualitative and quantitative PCR assays. The amplicon length and the applicable plasmid(s) are also given

2.1.3.3 Real time PCR setup using STP

A real time procedure has to be developed if existing method is not available for quantitation of a particular GMO.
CLO (partner #2) has developed, optimize and test different new real-time PCR assays for a serie of plasmid RM. This is a multistep procedure. First, event-specific primers are designed and their specificity was tested in a conventional, qualitative PCR. Second, a real-time PCR reaction is performed with the non-specific, ds-DNA binding dye SYBR Green I, to test again for the specificity of the reaction. Third, the primers are used in combination with a target-

specific TaqMan probe in a second real-time run. In both real-time experiments, the same dilution series of plasmid standards was used.

This procedure was followed for all targets constructed by CLO, however results will be shown and discussed here for a selected number of targets only, i.e. the event-specific sequences of Bt11, Bt176 and GA21 maize and of GT73 canola, as described in Taverniers et al. [13].

The specificity of the developed primer pairs was first tested qualitatively, by analyzing the transgenic event next to (1) the corresponding non-transformed, wild-type plant DNA, and (2) a series of other events: Bt11, Bt176, GA21, MON 810, T25, CBH-351 maize. Results on agarose gel of those specificity tests confirm that the developed, event-specific PCR primer sets are unique for the transgene event for which they were designed.

The sensitivity of the primers and the specificity for their target were also tested in real-time PCR by using the plasmid DNA calibrators. Per transgene event, a dilution series from 10^7 copies to one single copy of plasmid DNA was subject to a real-time PCR with SYBR Green I, including a dissociation step. Results for Bt11, Bt176 and GA21 maize and GT73 canola are shown in **Figure 4**. For each of the plasmid marker sets, amplification plots are shown together with the calculated calibration curve on the left as well as the amplicon’s melting peak on the right.

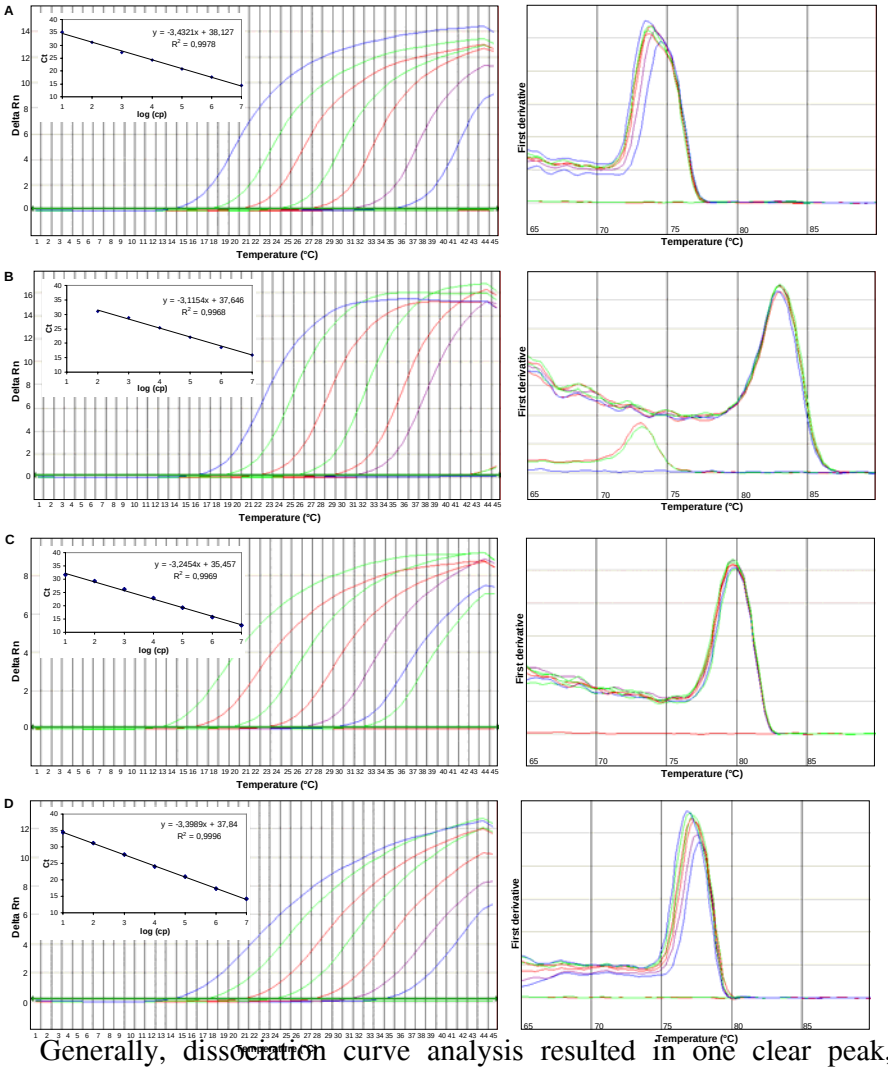


Figure 4: Amplification plots and corresponding calibration curves and melting peaks resulting from real-time PCR analysis with SYBR Green I, on plasmid DNA standards specific for transgene events Bt11 maize (**A**, 10-1E7 copies), Bt176 maize (**B**, 1E2-1E7 copies), GA21 maize (**C**, 10-1E7 copies) and GT73 canola (**D**, 10-1E7 copies). Fluorescence threshold values were set manually at 0.2 and baselines were set manually at 3-13. Standard curves were obtained with all points (10-107 cp) for Bt11, GA21 and GT73 and with one outlier (10 cp) for Bt176

Generally, dissociation curve analysis resulted in one clear peak, without formation of aspecific or primer-dimer products and with no observed signal for the NTC (flat curves). An

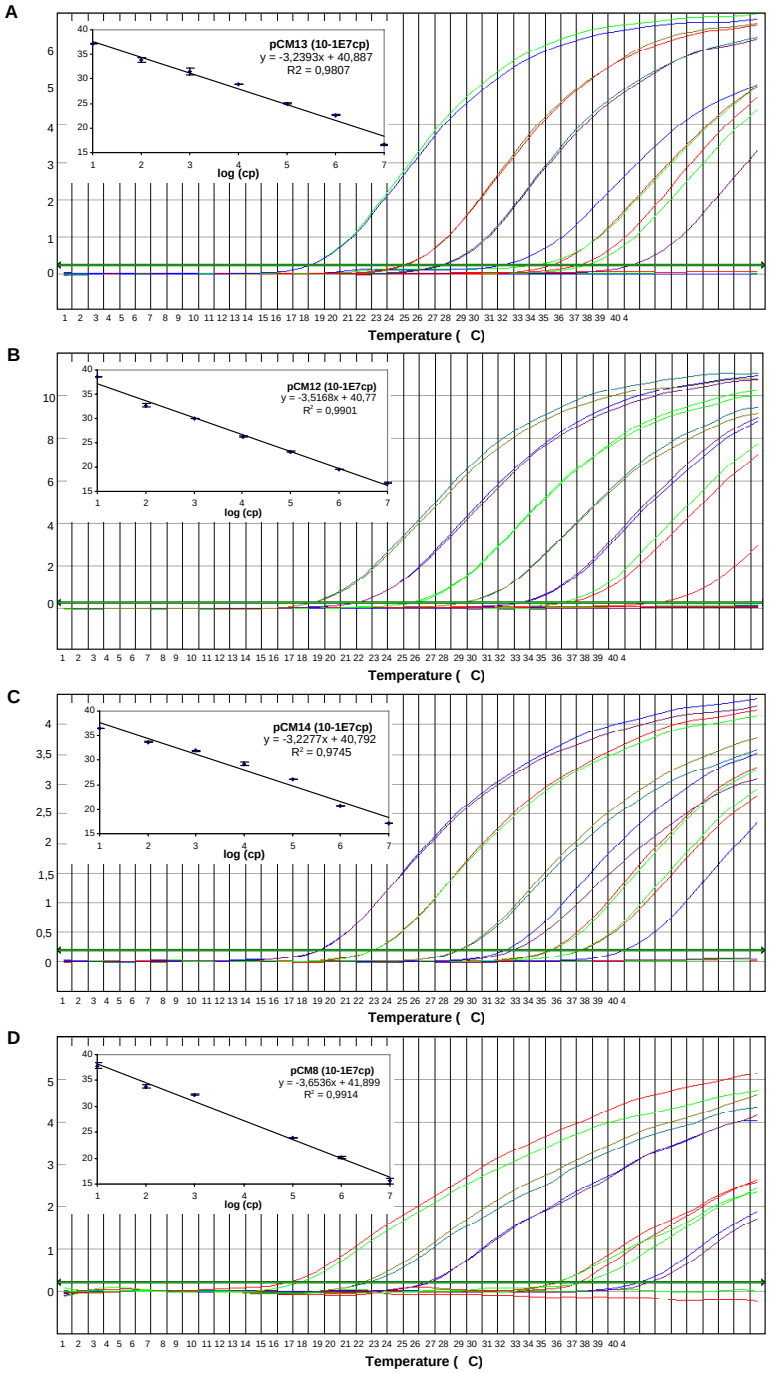
exception is the Bt176 event-specific assay, where a small primer-dimer peak was observed for the 10 cp and 1 cp samples (NTC is OK, see Fig. 4B). Mean melting temperatures with their standard deviations (SD) are (73.9 ± 0.3)°C for Bt11 maize (A), (83 ± 0.04)°C for Bt176 (B), (79.8 ± 0.1)°C for GA21 maize (C), and (77.3 ± 0.3)°C for GT73 canola (D) (Fig. 4).

These first experiments on the plasmid calibrators also allowed establishing calibration curves. Linear regression curves can be seen for each plasmid series on the graph of amplification plots. With correlation coefficients (R^2) of at least 0.99 and slopes between -3.1 and -3.4 (PCR amplification efficiencies between 0.968 and 1.102 respectively), it can be said that the curves are acceptable. This already indicates that plasmid DNA molecules are highly suitable to establish calibration curves for absolute quantification of a single target-locus in the range from 10^7 to 10 copies. Nevertheless, SYBR Green I is generally not usable for accurate quantification.

The same series of plasmid calibrators as used in the SGI assays (10-10^7 copies) was subjected to a TaqMan real-time PCR experiment. The objective was to check whether good calibration curves could be established, usable for specific absolute quantification purposes. **Figure 5** shows the amplification plots, resulting calibration curves and C_T values for the four event-specific plasmid calibrator series.

Amplification plots were obtained for calibrators from 10^7 down to 10 copies. The slopes for the four curves are -3.24, -3.52, -3.23 and -3.65 respectively, corresponding to PCR amplification efficiencies of 2.035, 1.92, 2.040 and 1.879 respectively. These efficiencies can be normalized and translated as follows: 98.23 %, 96.17 %, 98.01 % and 93.96 %. These values are acceptable, except for 93.96 %, which is too low. Values for Y-intercept vary between 40.77 and 41.90, which is quite close to the theoretical value of 40, representing the number of cycles, which is theoretically needed to amplify a single target copy. The correlation coefficients, finally, are acceptably high, varying between 0.975 and 0.991.

Figure 5 shows that it is possible to construct calibration curves with the plasmid calibrators, in the range from 10 to 10^7 copies. Duplicate analyses are highly reliable. An exception (no reliable results) forms the lowest calibration point used to establish the curve (10 cp or log(cp) = 1). Here, generally one of two replicates gives a signal while the other one results in a C_T value of 45 (negative). In other words, 10 cp samples are positive in 50 % of the measurements, while 1 cp generally is not detectable. It is important to remark that these findings are based on a single run, with two replicates only. From Fig. 5, high similarities are generally seen between C_T values for the same plasmid DNA concentration, over the different plasmid markers [13].



cp	log(cp)	Ct (1)	Ct (2)	Mean Ct	SD (Ct)
1	0	outlier	outlier	/	/
10	1	45	37.08	/	/
1.00E+02	2	33.59	34.17	33.88	0.41
1.00E+03	3	32	31.1	31.55	0.636
1.00E+04	4	28.88	outlier	28.88	/
1.00E+05	5	25.14	24.95	24.95	0.134
1.00E+06	6	22.67	22.55	22.61	0.085
1.00E+07	7	16.54	16.58	16.56	0.028

cp	log(cp)	Ct (1)	Ct (2)	Mean Ct	SD (Ct)
1	0	45	45	45	0
10	1	38.54	45	/	/
1.00E+02	2	32.93	32.41	32.67	0.368
1.00E+03	3	30.05	29.92	29.985	0.092
1.00E+04	4	26.33	26.27	26.3	0.042
1.00E+05	5	22.95	23.16	23.16	0.148
1.00E+06	6	19.49	19.47	19.48	0.014
1.00E+07	7	16.73	16.84	16.785	0.078

cp	log(cp)	Ct (1)	Ct (2)	Mean Ct	SD (Ct)
1	0	45	45	45	0
10	1	36.53	45	/	/
1.00E+02	2	33.79	33.71	33.75	0.057
1.00E+03	3	31.79	32.02	31.905	0.163
1.00E+04	4	28.95	29.51	29.23	0.396
1.00E+05	5	26.1	26.04	26.04	0.042
1.00E+06	6	20.56	20.57	20.565	0.007
1.00E+07	7	17.16	17.14	17.15	0.014

cp	log(cp)	Ct (1)	Ct (2)	Mean Ct	SD (Ct)
1	0	45	45	45	0
10	1	37.45	38.18	37.815	0.516
1.00E+02	2	33.64	34.05	33.845	0.29
1.00E+03	3	32.35	32.21	32.28	0.099
1.00E+04	4	outlier	outlier	/	/
1.00E+05	5	24.18	24.02	24.02	0.113
1.00E+06	6	20.28	20.03	20.155	0.177
1.00E+07	7	15.25	15.94	15.595	0.488

Figure 5: Calibration curves resulting from real-time PCR analysis with TaqMan chemistry, for 10-1E7 cp pDNA of (**A**) Bt11 maize (pCM13), with the following calibration curve settings: manual threshold of 0.237, manual baseline of 3-13 and outliers 1 cp (2 replicates) and 104 cp (1 replicate); (**B**) Bt176 maize (pCM12), with manual threshold of 0.279, manual baseline of 3-15 and no outliers; (**C**) GA21 maize (pCM14), with manual threshold of 0.2, automatically set baseline and no outliers; and (**D**) GT73 canola (pCM8), with manual threshold of 0.214, automatically set baseline and outlier 104 cp (2 replicates). “/” means not relevant in this case. Remark: points with a CT value of 45 are automatically excluded from the calibration curve.

Relative quantification of GMOs is possible by performing a double quantitative PCR: one amplifying a species-specific sequence and one targeting the event as such. This means that the absolute quantitative information obtained from the two reactions separately can be combined in order to calculate final percentages. By dividing the number of event-specific target copies by the number of taxon-specific target copies, and multiplying this number by 100, a relative percentage is obtained, expressed on the level of haploid genomes or DNA. The suitability of the plasmid DNA calibrators for relative quantification of GMOs was tested for Roundup Ready soybean. Dilution series from 10^6 down to 10 copies per PCR tube of the plasmids pAS104 and pAS106 were prepared as calibrators as described above. Genomic DNA was prepared from 0, 0.1, 0.5, 1 and 2 % Roundup Ready soybean CRMs (ERM^R-BF410a/f), to be used as unknown samples. TaqMan real-time PCR reactions were performed with the primers and probes listed in Table 2 (RRS-p-35S border system and lectin system). After the run, data were analyzed, separate calibration curves established for both systems (**Figure 6**), and relative percentages of RRS calculated, together with the standard deviation, % of relative standard deviation (% RSD) and % of error (**Table 6**).

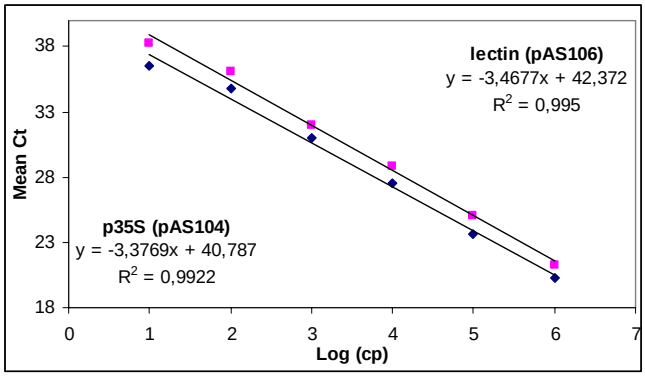


Figure 6: Calibration curves for 106-10 copies of the p-35S junction of RRS (pAS104, lower curve) and for 106-10 copies of the soybean lectin gene (pAS106)

Sample	Ct junction	Mean Ct junction	SD (Ct junction)	Ct lectin	Mean Ct lectin	SD (Ct lectin)	Cp junction	Mean cp junction	Cp lectin	Mean cp lectin	Conc (%)	Mean conc (%)	SD	%RSD	% error
NTC	45 45	**45**	0	45 45	**45**	0									
10	36,16 36,97	**36,57**	0,57	38,02 38,46	**38,24**	0,31									
100	34,97 34,55	**34,76**	0,3	36,59 35,56	**36,08**	0,73									
1000	31,31 30,67	**30,99**	0,45	45 32,02	**32,02**	9,18									
10000	27,81 27,34	**27,58**	0,33	28,79 28,82	**28,81**	0,02									
100000	23,35 23,85	**23,6**	0,35	24,92 25,1	**25,01**	0,13									
1000000	20,24 20,37	**20,31**	0,09	21,28 21,22	**21,25**	0,04									
0	36,41 37,64	**37,03**	0,87	26,76 27,48	**27,12**	0,51	19,76 8,54	**14,15**	31594,6 19593,6	**25594,1**	0,063 0,044	***0,054***	0,013	24,07	5,4
0,1	30,39 30,25	**30,32**	0,1	21,28 21,22	**21,25**	0,04	1194,23 1317,9	**1256,07**	1203256 1167851	**1185553**	0,099 0,11	***0,105***	0,008	7,62	5
0,5	29,52 29,68	**29,6**	0,11	22,54 22,69	**22,62**	0,11	2163,36 1949,07	**2056,22**	521604 472012	**496808**	0,41 0,41	***0,41***	0	0	18
1	28,06 27,79	**27,93**	0,19	22,13 22,57	**22,35**	0,31	5863,98 7074,68	**6469,33**	683685,8 511535,9	**597610,8**	0,86 1,38	***1,12***	0,37	33,04	12
2	27,65 27,07	**27,36**	0,41	23,23 22,53	**22,88**	0,49	7743,99 11515,18	**9629,59**	306759,7 330960,3	**318860**	2,52 3,48	***3***	0,68	22,67	50

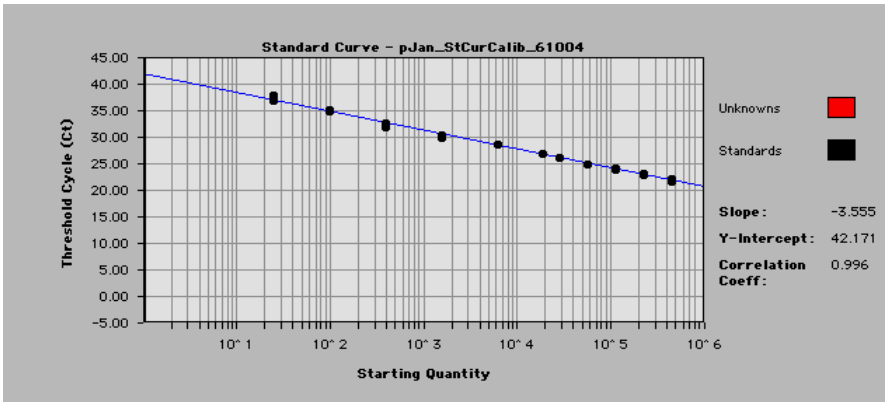
Table 6: Quantitative estimates of relative % of RRS in five Fluka CRM samples, obtained from measured CT values and absolute copy numbers for the endogenous *lectin* system and the event-specific target system. Standard deviations (SD), percentage relative standard deviations (% RSD) and % error values on the final relative RRS % are also given. ‘cp’= measured numbers of copies, based on the plasmid calibrators which are expressed as absolute copy numbers, and referring to the number of haploid genomes in the sample

Table 6 demonstrates that plasmid DNA solutions, containing absolute numbers of copies of the target sequences, allow to relatively quantify GMOs in percentages. Precise (mean % RSD of 17.48 %) and accurate (mean % error of 18.08 %) RRS estimates were obtained for five different RRS-containing genomic DNA samples in the range from 0 to 2 %. Bias (% error) relate to the expected values, which in this case are the certified reference values from the RRS CRMs. This experiment proofs that reliable quantification of GMOs is possible with plasmid DNA standards.
A remarkable observation from Table 6 is the positive result for 0 % RRS. A mean C_T value of 37.03 for the event-specific target PCR is obtained. This means that the calculated number of copies is significantly different from zero (mean value of 14.15 cp). The relative RRS content, 0.054 % is the result from the ratio of this number of copies to the total number of soybean-specific target copies (mean value of 25594.1 cp). As the NTC is negative (C_Ts of 45), no contamination occurred during the PCR. As a consequence, the 14.15 copies of GMO target may originate from a low level of contamination in the sample DNA. On its turn, this may be the result of repeated freezing, thawing and use of the DNA in an environment where GMO DNA is present.

2.1.3.4 pJANUS plasmids as calibrants for quantification of GMOs using real time PCR

In the framework of this project it was also proposed to develop and to pre-validate double target plasmids (the so called “pJanus”) in quantitative GMO analysis. In 2003, the WIV (partner #1) assessed the pJANUS plasmid" pENGL-03-006. This plasmid includes a fragment of the pat gene (GM transgenic marker) and a fragment of the zein gene (maize-specific marker). Two separate PCR reactions were performed for each marker. The plasmid system was then calibrated with the Bt11 CRM. When comparing copy numbers calculated for the plasmids with copy numbers calculated for the certified reference material a correction factor can be deduced. This approach developed initially by the JRC-Ispra (one of the non financed partner) has know to be systematically develop for constructing new pJanus plasmid GMO placed on the EU market by combining an event-specific amplicon and an ingredient specific amplicon.
Different strategies have been studied to join together in a unique plasmid the two fragments of interest. One of them consists to amplify two amplicon from STP using tailed primers to join them subsequently to a second PCR reaction. The resulting amplicon was cloned in the final pUC18 vector. The obtained plasmid is checked by restriction mapping and sequencing. As for STP, a scientific dossier containing all the useful information has to be produced for each constructed pJANUS plasmid.
The applicability for the use of the constructed pJANUS in real-time PCR analysis as standard has to be developed. The strategy consists to purify the plasmid after linearization and to quantify it using the PicoGreen dye. A serial dilution in TE buffer containing carrier DNA is performed. The dilution series can be used as calibrator to construct the standard curve in real-time PCR experiments. Working concentration of the dilution series has been calculated in order to encompass a hypothetical sample under analysis and the goodness of the dilution series has been assessed checking both cloned markers.
This technique has been applied to construct the pJanus RRS, dedicated to the quantitation of the transgenic Roundup Ready soybean. The pENGL-02-001 (containing part of the soybean lectin (Le1) gene) and the pAS104 (containing the E35S promoter/plant junction region) were used to construct the pJANUS RRS. Two separate reactions for each amplicon were carrying out to evaluate the linearity of the standard curves (**figure 7**).

A



B

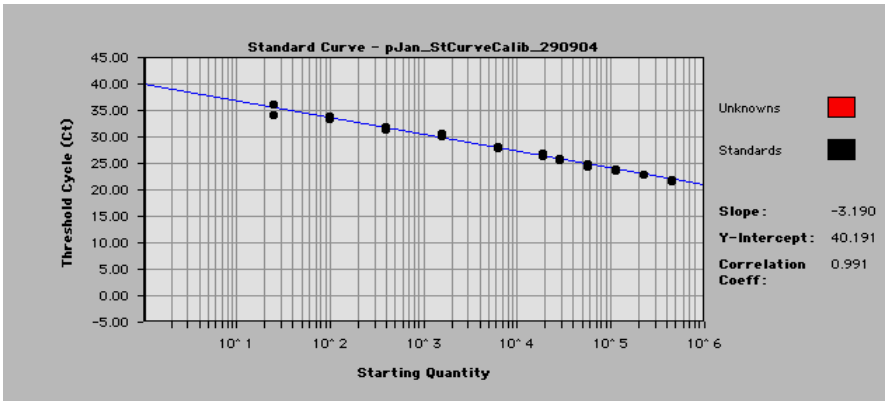


Figure 7: Standard curves obtained analyzing the pJanus RRS series with the lectin specific real-time PCR system (**A**) and with the transgene specific real-time PCR system (**B**).

The method developed is intended to be used as a general protocol for future pJanus-type plasmids. An inter-laboratory study in order to pre-validated a real-time method, coupled with the pJanus RRS standard curve, is foreseen. The organization, collection and the analysis of the results will be carried out in the SBB.

2.1.3.5 Applicability and commutability of plasmid DNA calibrators

In order to comply with current EU regulations concerning the mandatory labelling of GMOs and GMO-derived food and feed products with a minimum GMO content of 0.9 %, reliable quantitative methods are needed. On the basis of quantification lie the used calibrators or standards. An intense debate is currently ongoing with regard to the type of calibrator best suitable for GMO quantification. The main argument against plasmid molecules (compared to genomic DNA RMs) is the idea that these molecules would not “mimic” the genomic DNA extracted from CRM.
As a consequence of food and feed processing and of DNA extraction procedures, gDNA quality can be extremely poor, e.g. DNA may be degraded, and PCR inhibitors may be present. To the contrary, as plasmid DNA solutions contain purified templates without the risk for competition with ‘bulk’ genomic DNA sequences, these are very likely to be favoured in PCR reactions. At least, this is a theoretical assumption. Since the introduction of pDNA calibrators [12], the fear has been growing amongst GMO analysts that gDNA and pDNA would show different PCR amplification efficiencies and would therefore not be commutable.

For this reason, CLO (partner #2) performed a ‘commutability study’. For four different transgene events, plasmid and genomic DNA calibrators were prepared, containing absolute numbers of target sequences in dynamic ranges as wide as possible and similar for both types. Absolute standard curves were established and compared in terms of amplification efficiency, dynamic range and characteristics of the standard curve, and sensitivity.
In addition, an extensive comparison is made between single-target plasmid (STP), multiple-target plasmid (MTP) and genomic DNA standards. Quantification of GMOs is accomplished by means of a single delta C_T curve for the STP and genomic DNA standards, or by means of two absolute standard curves for the MTP calibrators. In this way, we are able to evaluate three different types of calibrator sets for GMOs, but also different approaches to set up standard curves and to obtain final relative GMO contents as results. For more details on the description and the results of this extensive comparative study, we refer to Taverniers et al., 2004a[14]. Here, a summary is provided.

In this study, plasmid and genomic DNA calibrators were compared for quantification of GMOs. A proof for commutability was obtained from calibration curves, set up with both types of standards and showing very similar characteristics. We also described three real-time duplex assays for GMO quantification.
Plasmid DNA calibrators were compared with the classical genomic DNA standards from the commercially available certified reference materials (CRMs). We have proven that, for duplex quantification based on a delta C_T curve, single-target plasmids (STPs) mixed with genomic pure soybean DNA with concentration levels from 50 to 0.75 % perform at least as well as genomic DNA samples from commercially available CRMs (5-0.1 %). The DNA calibrators allow quantification in a wider range and the different concentrations show a higher correlation compared to genomic DNA standards, which are independent DNA extractions.
One drawback of plasmid DNA calibrators is that they, as such, only contain the pure analyte and are not similar to real samples of interest. However, plasmid DNA RMs used in this study were made ‘matrix matching’ by spiking them in a background of genomic DNA. Another prerequisite for delta C_T methods is that equal PCR amplification efficiencies for both targets are obtained. Because of this drawback of delta C_T methods, we also developed a duplex quantitative method with plasmid DNA calibrators expressed in copy numbers. Optimal calibration curves were set up with multiple-target plasmid (MTP) DNA standards containing different DNA target sequences. In addition, quantitative results were delivered with high precision and accuracy. Summarized, plasmid DNA molecules containing multiple fragments next to each other show excellent performance in a real-time duplex PCR.
Throughout this study, a commercial kit was used with optimized reagent concentrations for duplex PCR but targeting (next to a sequence of the endogenous lectin gene) only the promotor 35S. The primers and probe specific for the p-35S element could be used in combination with the MTP fragments developed by the Japanese researchers as well as with our STPs containing the p-35S T-DNA/plant junction fragment of Roundup Ready soybean. However, because we stuck to the use of this commercial kit, reactions were only screening and not event-specific. Future developments of real-time duplex PCR methods could aim at transformation event-specific sequences, such as the p-35S/plant border of Roundup Ready soybean present in the STP used. Such a junction, also called ‘cross border region’ or ‘edge fragment’, or any other event-specific fragment such as a rearrangement, is the only unique signature of a transformation event. As junctions of different commercialized GMOs have been characterized and cloned, event-specific duplex quantification assays could be developed with these plasmid DNA calibrators [14].

2.1.3.6 Discussion and conclusions

GMO quantification and the interpretation of threshold percentages are topics, which were addressed in particular within this project. The units of measurement and expression of GMO contents are closely linked to the type of calibrators used for quantification at the analytical level. More in particular, the unit of the used quantitative standards should be consistent with the unit of expression. In practice, if GMO contents are to be expressed as a ratio of genome copy numbers, this requires calibration standards to be expressed also in copy numbers. In 2001, the first publication on event-specific plasmid DNA standards, tested for Roundup Ready soybean, appeared [12]. In the same year, we introduced the new concept at a plenary ENGL meeting. The idea to construct plasmid DNA markers containing GMO-specific as well as taxon-specific DNA sequence targets, has been worked out within this research project. ENGL has taken over the initiative to develop a database of plasmid markers for GMOs, while implementation is a role of the Belgian Network of GMO Laboratories (BNGL).
Partner #1, #2 and #5 have developed plasmid markers for a series of commercialized GMOs. For each plasmid marker they constructed, partner #2 designed a highly specific primer pair and a fluorescent TaqMan probe, to be used in qualitative as well as quantitative real-time PCR applications. Quantification is made possible by combining a GMO-specific PCR assay with an endogenous, species-specific PCR. This ‘double quantification’ allows to calculate a relative percentage, expressed on a haploid genome (copy number) basis. The developed PCR-based methods for event-specific identification and quantification allow testing of GM seeds, food and feed products, and thus horizontal implementation of EU threshold-regulations. In addition, we have shown the usefulness of this new type of calibrators, and the perfect commutability with other types of DNA, such as genomic DNA from routine samples.

2.1.4 Influence of the genetic structure of seeds on real-time PCR GMO quantification

2.1.4.1

Of special concern in the frame of GMO expression units and quantification, is quantification of GM seeds. Despite the fact that still, no specific legislative framework has been established for the labelling and analysis of transgenic seeds, it is generally assumed that GM contents in seed lots should be expressed on a seed-to-seed basis. If a seed/seed percentage is to be the unit of expression of GMO contents in seed samples, one can immediately conclude from this that real-time PCR is not the suitable technique for quantification. This claim is justified by taking into account the specific nature and structure of a seed. A maize kernel consists of an embryo (12 %), an endosperm (82 %) and a seed coat or tegument (6 %) (**Figure 8**). Seeds are formed through double fusion of nuclei. First, a haploid maternal nucleus and a haploid male nucleus fuse to form the diploid embryo (2n). Second, the triploid (3n) endosperm results from the fusion of two material polar nuclei with one sperm nucleus. Intensive mitosis, followed by endoreduplication of the DNA result in endosperm development. Embryo and endosperm are enveloped by a diploid tegument (2n), originating from the mother only [15-17].

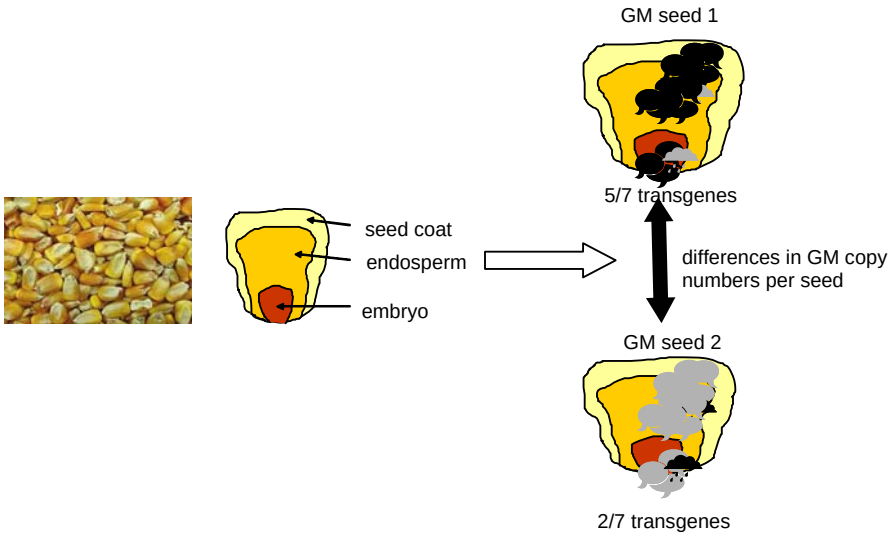


Figure 8: Conformational structure of a seed, here illustrated for a corn seed (grain), and the implications on quantification and expression units for GMOs. A % GMO expressed in genomes is different from a % GMO expressed in numbers of seeds. Quantification of GM DNA in seeds is dependent of the origin of the seed, more in particular of the transgenic parent (black:transgene allele, grey: wild type allele)

Transgenic maize kernels are hemizygous, resulting from the crossing of a homozygous transgenic parent with a non-transgenic one. Because male and female genomes do not equally contribute to the maize kernel, the relative content of a transgene genome in heterozygotes will be dependent of its inheritance. This effect would be negligible if one could assume that the kernel’s total DNA mainly originates from the embryo and if only this embryo part would be used for DNA extraction. However, as whole seeds are milled and used in DNA extraction and as endosperm cells can contain up to 690C – with C being the unreplicated, haploid DNA content per nucleus [18]– this is not the case. Taking reckon with the differences in endosperm ploidy, and derived from (1) a weight endosperm:embryo ratio of 20:1 and (2) a DNA content endosperm:embryo ratio of 10:1, Papazova et al. [19] concluded that, in the case of maize, the embryo’s DNA content is about twice that of the endosperm.

Within this project, some experiments were performed both by CLO (partner #2) and CRA (partner #5) in order to test the theoretical assumptions in practice, on real seed samples.

For CLO, in a first stage, theoretical calculations were done for maize, based on the above described characteristics of seeds. Papazova et al. [19] claim that, depending on differences in DNA quantities between endosperm and embryo, in combination with differences in origin of the respective genomes, for each specific species, a ‘conversion factor’ should be used to make a translation from genome percentage to seed percentage. The author’s suggestion is to use a ‘safety threshold’, in case if real-time PCR is used to quantify the GMO content in a seed lot and if this content is to be expressed on a seed basis. For maize, an individual seed is transgenic if 11 of the 32 genomes are transgenic. If a regulatory threshold of 0.5 % is handled for seeds, this seed/seed ratio comes down to a ‘working lower threshold’ for real-time PCR of 0.1718 %, expressed in genomes. To summarize, the specific composition of a seed kernel, the way of inheritance and the ploidy level support the discordance between a GMO % in genomes and a GMO % in numbers of seeds [17,19].

In a second stage, those findings were tested by CLO for transgenic T25 maize seeds. For different types of seeds – seeds that are heterozygous for the transgene locus, homozygous, or a mixture of both – a genome/genome % GMO was measured and compared with the well-

known seed/seed % GMO. Generally, the estimated genome/genome percentages were lower than the seed/seed percentages.

CRA (partner #5) also evaluate the assumption of the “conversion factor”. The two main calibration systems (percentage of mass fraction of genetically modified (GM) material in flour based on use of flours made of ground kernels and percentage of transgene copy numbers now generally obtained through use of plasmids in known amounts) were assayed on the GM maize MON810 as model. As already mentioned, due to biological reasons linked to zygosity and ploidy of the several plant tissues (especially in kernels), both calibration systems will not necessarily come up with identical figures but are supposed to be linked through a conversion factor (Cf) also named Cv for coefficient value by Japanese teams [20,21]. The comparison is performed to establish what is the conversion factor between the expression of GMO content in % of mass fractions (IRMM MON810 standards) and the % of GM copy number obtained through plasmids (Nippon gene Multi Target Plasmid) as reference material.

Standards used for the experiments are (1) the IRMM reference material (GMO maize powder set for MON810, ref. IRMM-413) from two different batches of reference powders (batches called DP1 and DP2 for *Dry powder*) with the complete range of certified contents (0.1%; 0.5%; 1.0%; 2.0% and 5.0%) giving in % the mass fractions of heterozygous MON810 maize powder present in the whole powder; (2) two batches (MTP1 and MTP2, MTP for Multi Target Plasmid) of a set of vials with calibrated amounts of a suitable multiple target plasmid at the respective amounts of 20, 150, 1 500, 20 000 and 250 000 copies per 2,5 µl produced by Nippon gene and distributed in Belgium by Diagenode (GM Maize Detection Plasmid Set, ref. 319-04981). These plasmids contain both a fragment of a maize endogenous gene (*zSSIIb* target) and a fragment of MON810 maize construct (MON810 target).

The comparison samples (CS1 and CS2) to be quantified with the two calibration systems are two maize flours containing respectively 1.3% (CS1) and 0.7% (CS2) expressed in mass fractions. Each of the reference flours and also the comparison samples were submitted to DNA extraction with the CTAB method according to the European standard prEN ISO 21571[22]. For this extracted material, approximately 20 000 copies of maize haploid genome equivalents were used in each PCR. The MTP vials were ready to use. Four PCR plates were run containing always the comparison samples and one of the four combinations between an IRMM standard batch and a MTP set batch (i.e. MTP1/DP1, MTP1/DP2, MTP2/DP1 and MTP2/DP2).

Real-time PCR were performed on an ABI Prism® 7000 according to conditions that had been optimised [20] and validated [21]. Every calibration point and each comparison sample used on a plate were replicated four times per target. Analysis of the resulting amplification data was performed in “auto Ct” mode [23]. This automatically assigns a baseline per well and calculates a fluorescence threshold per target. Calibration curves were obtained either through ΔCt method for the IRMM standards or by separate curves per target for the MTP set of points with use of their respective Ct values. For the four runs the content of MON810 in both comparison samples were determined and the copy number ratio for each calibration point of the IRMM standards was calculated. When calibration is done with the plasmids the final result is expressed as hundred times the ratio between the copy number of MON810 target to the copy number of the maize specific target. With the reference flours as standards the final percentage represents the ratio between the mass of heterozygous MON810 flour (or at least considered as such in the sample) to the total mass of maize flour in which the MON810 material is contained (See equations 1,2,3 for the calculations of GMO amounts).

$$\text{Copy number (of a specific target)} = n^{[(Ct-b)/a]} \quad (1)$$

$$\text{GMO amount in copy number (\%)} = \frac{\text{Copy number of GM marker target}}{\text{Copy Number of endogenous target}} \text{ X } 100 \quad (2)$$

$$\text{GMO amount in mass fraction (\%)} = n^{[(\Delta Ct-b)/a)]} \quad (3)$$

With : n = base of the logarithm which is used in the calculations
b = intercept of the standard curve
a = slope of the standard curve
Ct = Cycle threshold = fractional cycle number at which the fluorescence passes the fixed threshold
ΔCt = GM marker target Ct - endogenous target Ct

The correction factor (Cf) was determined with the help of figures obtained in the two reference systems for the comparison samples (equation 4) but also on each calibration point of the IRMM standard (equation 5).

$$Cf = \frac{\text{GMO amount in copy number (as calculated in equation 2 with MTP calibrators)}}{\text{GMO amount in mass fraction (as calculated in equation 3 with DP calibrators)}} \quad (4)$$

$$Cf = \frac{\text{GMO amount in copy number (as calculated in equation 2 with MTP calibrators)}}{\text{Certified GMO amount in mass fraction}} \quad (5)$$

As a result, the derived conversion factors were respectively (0.60 ± 0.04) and (0.62 ± 0.06) for the comparison samples CS1 and CS2. Similar values ranging between (0.55 ± 0.19) and (0.74 ± 0.19) were obtained for each calibration point of the IRMM standards **(Table 7)**

Run -->	1	2	3	4	Variability		
Calibrators -->	MTP1-DP1	MTP2-DP1	MTP1-DP2	MTP2-DP2			
Sample	Cf values for IRMM standards (CRM)				Mean Cf	SD Cf	RSD Cf (%)
CRM 0.1%	0.56	0.64	0.78	0.99	0.74	0.19	25.56
CRM 0.5%	0.57	0.61	0.71	0.59	0.62	0.06	10.08
CRM 1.0%	0.52	0.66	0.64	0.75	0.64	0.10	14.90
CRM 2.0%	0.61	0.57	0.63	0.70	0.63	0.06	9.05
CRM 5.0%	0.55	0.55	0.55	0.56	0.55	0.00	0.90
Sample	Cf values for Comparison Samples				Mean Cf	SD Cf	RSD Cf (%)
CS1 (1.3%)	0.56	0.58	0.62	0.65	0.60	0.04	6.69
CS2 (0.7%)	0.56	0.60	0.65	0.69	0.62	0.06	9.17

Table 7: Cf values calculated on each IRMM standard (CRM) and comparison samples (CS1 and CS2)

Although results in this experiment are consistent they don’t met the figures published by Japanese teams [20, 21] for single kernels (Cf of about 0.38 and 0.42). However the Japanese teams never determined the Cf on the IRMM standards. The origin of this discrepancy might be linked to the parental origin of the MON810 trait (paternal for the Japanese material, maternal for the IRMM CRM). Noteworthy too is the fact that a technical document joined to IRMM standards [24] gives a measure of the number of MON810 DNA relative to total maize DNA in haploid genome equivalents for each IRMM MON810 standard. Conversion factors calculated from these data are grossly comparable to our results. These results concerning the conversion factor between the two expressions of GM MON810 maize content were communicated at a scientific meeting held in the Netherlands [25].

2.1.4.2 Conclusions

Both work in the evaluation of a “conversion factor” demonstrate the difficulty to get a common and reliable value.
CLO (partner #2) demonstrates that, as heterozygous seeds differ in their GMO content, the conversion factor from genome/genome to seed/seed percentage cannot be determined equivocally. In their experiments the genome/genome percentage was estimated corresponding to certain seed/seed percentages. CLO proposed to define a working threshold for real-time PCR, corresponding to a given seed/seed threshold. The lower working threshold for real-time PCR corresponds to the case of heterozygous seeds with paternal inheritance of the transgene in the seed sample. Therefore, if the obtained genome/genome percentage is below the real-time PCR working threshold, this will guarantee that the seed/seed percentage in under the threshold for seeds and the sample does not need to be labelled [Papazova et al., 2005].
CRA (partner #5) gives evidence that at least on maize, the concept of conversion factor should be handled with great caution if the aim is to convert copy number ratios in mass fractions, considering the several different conversion factors obtained with MON810 maize. For the sake of harmonization of results between laboratories, it seems much more appropriate to express results in terms of copy number of a well selected GM marker per haploid genome equivalent as this is basically what real time PCR is able to measure. The recommendation 2004/787/EC [7] of the European Commission is consistent with this last statement.

2.2 THEME 2: QUALITATIVE BIOCHIP FOR GMO DETECTION (PARTNERS #1 AND #3)

2.2.1 Introduction

Regarding the increasing diversity of GM plants arising on the world market, the « ideal » analytical tool should perform in a one shot experiment the screening of all GM plant markers. This technique of choice does not exist but we have taken advantage of a patented Belgian technology of one partner (promoter #3) that allows a mass screening of agro-food products in a fast, cheap and reliable manner: the DNA biochip technology. The principle of this technique relies on the specific interaction of a PCR product to a single strand DNA (so-called capture probe) covalently linked on a solid surface. It is possible to screen one sample for different GM plant while reducing the number of PCR reactions, thus reducing the cost of an analysis.

This technology already exists for gene expression studies and screening of point mutation in gene fragment. The adaptation of the technology to the GM plant market was a major goal for the project. Qualitative GMO chip is a middle size solution to cope with GMO diversity and the increasing lack of efficient GMO screening tools using the so-called "universal analytes" such as 35S promoter and NOS terminator.

During the first part of the project, consensus primers have been selected for the GMO detection and identification. These primers allow the amplification of the same element in different GMO. The events are then identified on the biochip according to their internal sequence.

In 2002, FUNDP (partner #3) designed a GMObiochip allowing the detection of 8 GMO events (Bt 11, Bt 176, MON 810, GA 21, T 25, T45, Topas 19/2 and GTS 40-3-2) by the mean of three primers pairs selected to amplify three different genes often present in the GMO: PAT, Cry1Ab and EPSPS. Three controls were present on the design : a fixation control to check for spotting efficiency, a positive hybridization control and a negative hybridization control to check for non specific hybridizations. The specificity of each probe spotted on the biochip could be demonstrated after hybridization of PCR products resulting from a simplex reaction. However, T25, T45 and Topas 19/2 hybridize to the same probe due to similarity of the amplicons amplify from these events.

This initial chip had to be upgraded for species specific detection. In 2004, FUNDP set a new design for the qualitative biochip. Next to the GMO identification probes, the following element were added to the chip:

1. Three species specific capture probes: These capture probes allow the identification of soybean, maize and canola. This identification system is based on the amplification of a plant specific gene with one specific primer pair followed by hybridization on specific capture probes.
2. A universal capture probe for plant detection. The test is based on the amplification of a plant specific marker with consensus primers followed by hybridization on a universal capture probe.
3. Food is a complex matrix and contains compounds as fat, salts, proteins and/or polysaccharides, which can inhibit PCR amplification. Then, the efficiency of PCR amplification may vary between samples depending on the presence of PCR inhibitors, leading sometimes to false negative results. To overcome this drawback, an internal standard plasmid has been constructed. This plasmid can also be amplified with the plant consensus primers and is added in each sample. A specific capture probe for this positive PCR control is present on the GMOchip.

2.2.2 Methodology and results

The general principle of the test as it has been developed can be divided in different steps:

1. DNA extraction from food samples.
2. Amplification and labelling of genetic markers often present in GMO. In order to reduce the number of PCR to perform, multiplexes PCR using consensus primers have been developed. During the PCR, amplicons are labelled with biotin by the mean of biotinylated deoxy-nucleotide triphosphate (dNTP).
3. Hybridization of the PCR products on their complementary capture probes which are grafted on a chemically treated glass slide.
4. Detection of the hybridized amplicons by a colorimetric technique. The biotin molecules are recognised by an antibiotin-gold conjugate which catalyses a silver salt precipitation. These silver spots can therefore be analysed and quantified by a colorimetric scanner.

In table 8 are indicated the names of the different primers pairs used in the multiplex reaction, the genes from which they have been selected and the GM events or plant species containing the target which are amplified with these primers.

multiplex PCR	Primers pair	Gene	Size of the amplicon	GMO
1	VPpat5-VPpat6	PAT	139 bp	T25, Bt11, T45, Topas 19/2
	VPcry3-VPcry4	CryAb	111 bp	Bt11, Bt176, Mon810
	VPepsps5-VPepsps VPepsps16	EPSPS	104 bp 255 bp	GA21, RRS
2	VPinv1-VPin2	invertase	104 bp	maize
	OPsoya1-OPsoya2	lectin	178 bp	soybean
	OPrape3-OPrape2	cruciferin	95 bp	rapeseed
	VPrbcl1-VPrbcl2	ribulose biphosphate carboxylase	95 bp 183 bp (PCR ctl)	all plant species and positiv PCR control

Table 8: Primers pairs used in multiplex PCR to amplify genetic sequences present either in GMOs or plant species

As shown in table 8, two multiplex PCRs are necessary for the amplification of the different GMO and controls. After the amplification step, both PCR products are hybridized on the same array.

The design of the GMO biochip is represented on the **figure 9**. Each capture probe is spotted in triplicate. The array is vertically divided in three parts: columns 2-4, 5-7 and 8-10. The first column is spotted with detection control in order to place the array in the right direction.

Horizontally, the rows 1, 2, 7 and 8 are spotted with a concentration curve of detection controls. Rows 9 to 12 are spotted with negative hybridization controls and a concentration curve of the positive hybridization control. The specific capture probes are spotted on rows 3 to 6 and their specificity is represented in **table 9**.

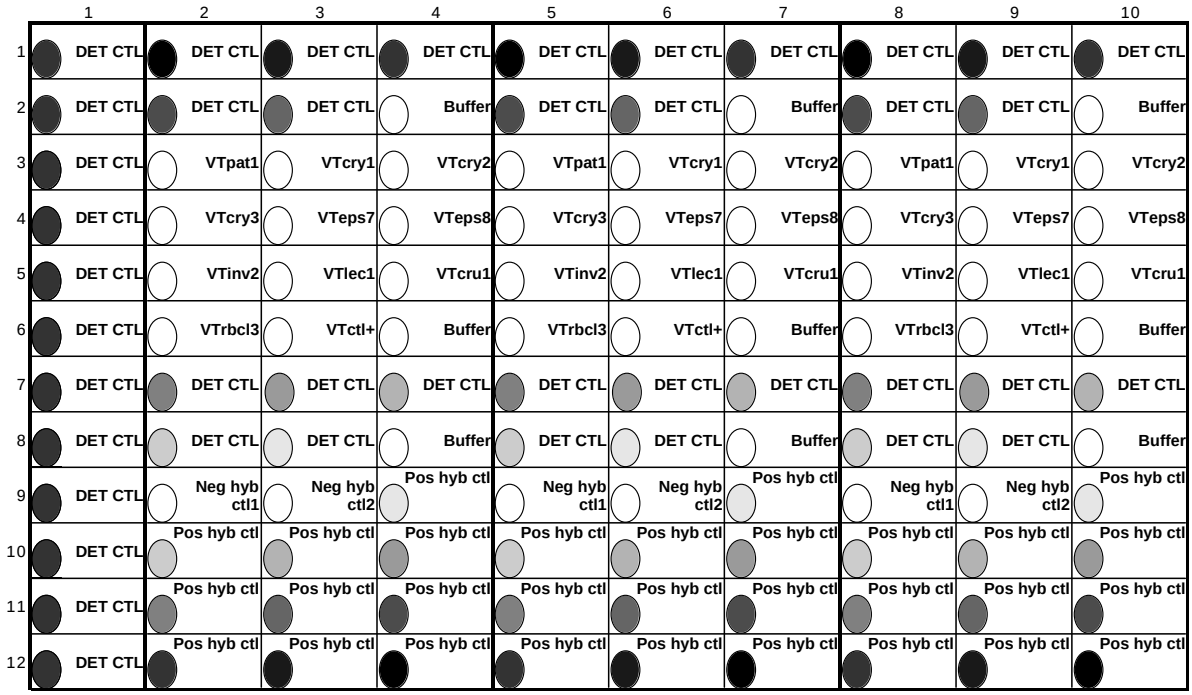


Figure 9: Design of the Biochips for the qualitative detection and identification of transgenic events T25 (VTpat1), T45 (VTpat1), Topas 19/2 (VTpat1), Bt176 (VTcry1), Mon810 (VTcry2), Bt11 (VTcry3 et VTpat1), GA21 (VTeps7) and RRS (VTeps8). The chips also allow the identification of the following plant species: maize (VTinv2), soybean (VTlec1) and oilseed rape (VTcru1). The VTrbcl3 probe is a general detector for plant. The VTctl+ is a positive PCR control for the internal standard plasmid.

VTpat1	T25, Bt11, T45, Topas 19/2
VTcry1	Bt176
VTcry2	Mon810
VTcry3	Bt11
VTeps7	GA21
VTeps8	RRS
VTinv2	maize
VTlec1	soybean
VTcru1	rapeseed
VTrbcl3	all plant species
VTctl+	positive PCR control

Table 9 : Names and specificity of the different capture probes spotted on the qualitative GMO identification biochip

The **figure 10** demonstrates that each GMO tested on the GMOchip hybridize to its specific probe. However, the positive PCR control linked to the amplificaion of the plasmid (spot VTctl+) is not always positive although the PCR control has been added in the mix. This should be a consequence a competition between the amplification of the rbcl gene naturally present in plant species and the positive PCR control due to the fact that, since they are amplified with the same primers. However, the result on the VTctl+ capture probe is particularly of interest in absence of other signals.
The universal capture probe VTrbcl3 gives also a positive answer for other plant species as shown for sugar beet, rice and tomato.

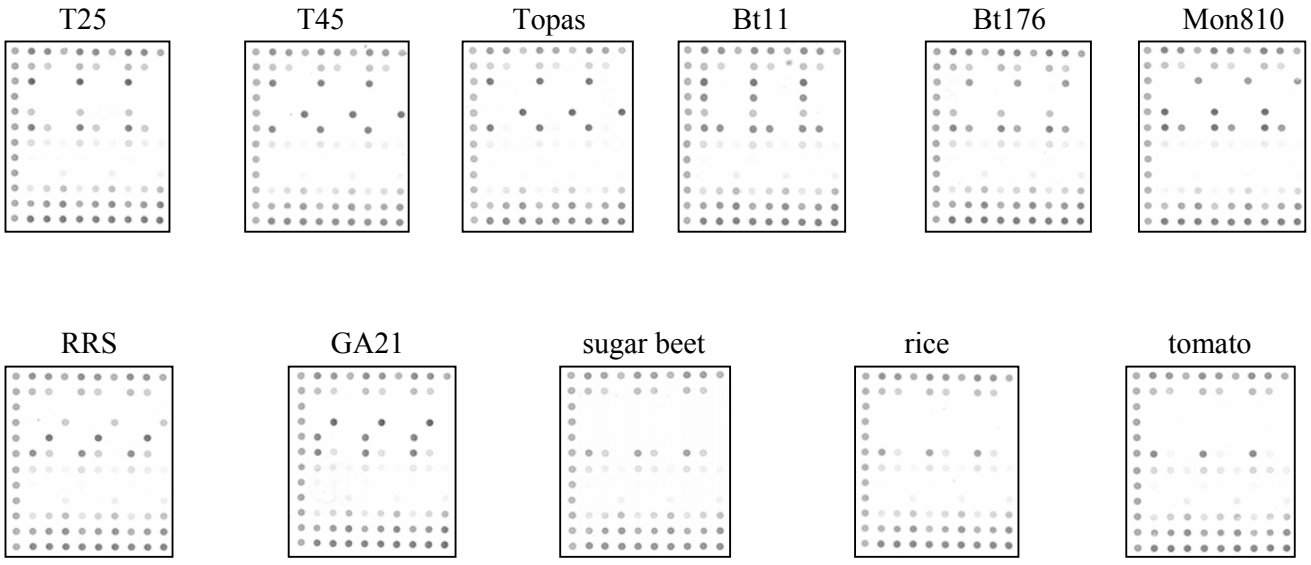
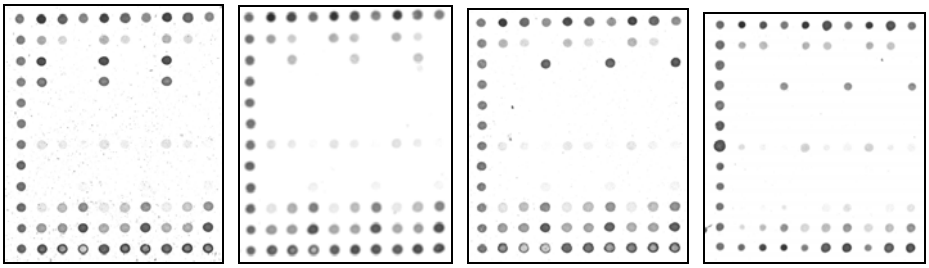


Figure 10: Hybridisation patterns of the different events after amplification with the two multiplexes PCR

The limit of detection (LOD) has been tested using certified reference material from IRMM (dried powder) for Bt11, Bt176, Mon810 and RRS. For these GMO, the GMO biochip can at least detect the presence of 0,1% (weight to weight) if 100 ng of DNA are engaged in the PCR. The same results have been observed for T25, T45 and Topas 19/2. For these events, the tests have been performed on material from Bayer. The biochip has been finalised in this way within the FUNDP.

The first validation using CRM was performed successfully by the FUNDP (partner #3). The technology was transferred to the ISP/WIV (partner #1). Reiteration of the experiment using certified reference material from IRMM (Bt 11, Bt 176, Mon 810 and RRS) allows the confirmation of the initial results. However, the full validation of the GMO biochips was foreseen with food/feed samples by the ISP/WIV. In this context, the cost for routine analysis of sample was evaluated and the quality-price ratio was not in the favour of the biochip if compared to the classical qualitative PCR. The main reason is the high cost of the biotinyled dCTP and dATP used for the detection system of the GMO biochip.

Biotinyled primers (one biotine molecule bound on the 5’ end) are a cheaper alternative to these biotinylated dNTPs. Preliminary tests have been performed at ISP/WIV on certified reference material for Bt11, Bt176, Mon 810 and RRS in order to evaluate the sensitivity of the qualitative GMO biochip subsequently to hybridization of PCR products amplified with biotinyled primers. As in the case of biotinyled nucleotides, theses four events were specifically detected on the biochips. For all these events, the LOD is set at 0,1% GMO content when 100ng of DNA were engaged in the PCR (**figure 11**).



Bt 11 0,1% Bt 176 0,1% Mon 810 0,1% RRS 0,1%

Figure 11: Hybridization patterns for Bt 11, Bt 176, Mon 810 and RRS after amplification with biotinylated primers set.

2.2.3 Conclusion

The development process of the qualitative detection GMO biochip had been performed. It was demonstrated, using reference material, that 0,1% GMO content could be detected when 100ng of DNA were engaged in the PCR. However some settings have to be performed, noteworthy with biotinylated primers before proceeding to the full validation of the biochip with food/feed samples.

2.3 THEME 3: ANALYTICAL CONTROL OF PARAMETERS INFLUENCING GMOS QUANTITATION (PARTNERS #4 AND #5): CHARACTERIZATION OF DNA EXTRACTS (TASK C2 UNDER THEME 3)

2.3.1 Introduction

One of the major problems regarding detection of transgenic DNA (or other targets) is the "variable quality" of extracted DNA. Damaged DNA, residual impurities or insufficient yields will totally or partially prevent DNA amplification, preventing and impairing real-time PCR. Moreover, lack of purity can lead to high variability.
Two groups were implicated in this task. The results obtained by these two groups are presented separately for an easier reading. Although several overlaps are present, in particular in the study of PCR inhibitors. Following abbreviations will be used throughout this part: PC stands for Phenol-Chloroform method, CTAB stands for CTAB extraction method, PFF corresponds to the Wizard® Magnetic DNA Purification System for Food of Promega combined with King Fisher, KF being the Thermolifescience extraction kit combined with King Fisher.

2.3.2 Analyse of "DNA quality" (Partner #4)

The main task of this group was to assess "DNA quality" by means of "chemical descriptors" and to correlate these with real-time PCR curves. Ultimately reliable and reproducible real-time PCR measurements should lead to trustful quantification of GMO-specific and species-specific markers in samples of good, medium and bad quality.
Analysis of DNA requires pure genomic DNA or plasmids. Custom extraction procedures using phenol/chloroform or CTAB and different commercially available extraction kits were evaluated.

2.3.2.1 Objectives

The first task consisted in choosing a model matrix from which DNA could be extracted as well as defining different extraction methods for obtaining genomic DNA of "variable quality" (Partner #4 and #5). The second task, analytical chemical methods were tested and optimised to separate DNA from any contaminating molecule (Partner #4).
Task three involved selection of matrices of variable composition (Partner #4 and #5) from which DNA was extracted according to different extraction procedures (partner #5). Subsequently these extracts were analyzed by real-time PCR (partner #5) and HPLC (partner #4) according to the methodology set up in task 2.
In the final task quantification methods of genomic DNA were evaluated and we looked also for the presence of PCR inhibitors in genomic DNA extracts (Partner #4).

2.3.2.2 Choice of model matrix and extraction procedures

In agreement with partner #5 (promotor Gilbert Berben, CRA, Gembloux) soybean meal was chosen as model matrix based on previous experience. DNA detection was done by amplification of a fragment of the LE1 gene (NCBI K00821, 2152 bp) coding for the soybean lectin (agglutinin) precursor (SwissProt entry P05046).
Initially, four different extraction protocols were used (see Material and Methods in attached document). Two were commercial kits: High Pure PCR Template kit (Roche, HP-extract) and the TLSc-DNA kit in combination with the King Fisher robot (ThermoLifeSciences, KF-

extract). The two other methods were phenol/chloroform and CTAB mediated extraction methods. Only CTAB extraction includes an RNase-digestion step.
All extractions were carried out by partner #5 (CRA, Gembloux). Apart from genomic soybean DNA extracts, we have also used a CsCl-purified plasmid containing a fragment of the LE1 gene (pLec), as well as different DNA standards or markers such as sheared salmon sperm DNA (fragments < 2000 bp, Invitrogen), □ phage DNA, digested with HindIII (Invitrogen), unshared genomic DNA (Roche) and high molecular weight markers (Invitrogen).
Quantifications were carried out spectrophotometrically, by measuring absorbance at 260 nm. Extraction yields, expressed as µg DNA per mg soybean meal, were as follows: 2,0 for the PC extract, 0,18 for HP-extract and 0,19 for KF extract

2.3.2.3 Analytical separation of DNA extracts by HPLC

High Pressure Liquid Chromatography (HPLC) seemed most suitable to purify genomic DNA. Separation of PCR amplification products, restriction fragments or oligonucleotides have been abundantly described in literature. Anion exchange chromatography (AEC) is most often used as nucleic acids are negatively charged over a wide pH range. Although purification of genomic DNA by HPLC is poorly described, AEC offered the best perspectives. Other purification options such as reversed-phase chromatography or size-exclusion chromatography have been briefly investigated but discarded after preliminary attempts.
A photo-diode-array (PDA) detector recorded the UV-spectral data of all components. Separation of genomic DNA from residual components was carried out on four different AEC columns: Protein Pak DEAE 15HR and 8HR, Gen Pak Fax (all from Waters) and DNA Pac PA100 (Dionex). Different conditions tested are described hereafter. Unless specified, we used a linear salt-gradient from solution A (25 mM Tris, 1 mM EDTA, pH 8) to B (25 mM Tris, 1 mM EDTA, 1M KCl, pH 8). For more details on these columns we refer to the SSTC intermediate report 2002 (contract CP42/322).

2.3.2.3.1 Separation on Protein-Pak DEAE 15 HR and 8 HR columns (Waters)

The Protein-Pak™ HR Ion-Exchange Glass Columns (Waters) are designed for separation of proteins. Packing material is based on rigid, hydrophilic, polymethacrylate particles with large 1000-Å pores. Beads are charged with functional DEAE (diethylaminoethyl) groups.
Three genomic DNA extracts as well as salmon sperm DNA, the □ HindIII digest and the pLec plasmid were injected on both columns. Absorbance was measured at 260 nm and flow rate was set at 0.5 ml.min^{-1}. Three main observations could be made.
First, the 15 HR column (15 µ beads) showed very broad peaks indicating poor resolution. The 8 HR column (8 µ beads) showed narrower peaks, but there was no separation (**figure 12**, 25 µg λ/Hind III fragments on 15 HR and 8 HR). Lambda phage DNA, digested with Hind III, gives a mixture of 7 different fragments varying in size from 23 kb down to 525 bp. None of the fragments could be isolated using either type of column.

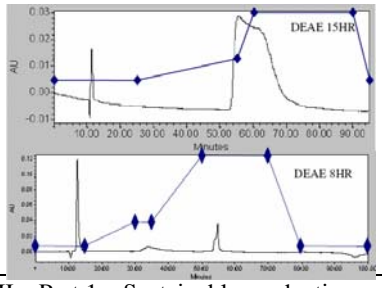


Figure 12: Chromatography of 25 µg λ/Hind III digest (Invitrogen) on DEAE 15 HR (top) and DEAE 8 HR (bottom). Elution gradient on DEAE 15 HR : 40% KCl to 100% after 60 minutes. Elution gradient of DEAE 8 HR : 20% KCl to 100 % after 50 minutes

Similarly, no resolution was observed with genomic DNA extracts of soybean meal.
Although the introduction of a step gradient allowed elution of extracts in several peaks, DNA eluted over a large time period, starting at Rt 41, Rt 40 and Rt 50 for KF- , HP- and PC-extracts, respectively (**figure 13**). PCR on HPLC fractions were adapted to avoid inhibition by excessive salt concentrations. Practically DNA amplification reactions were carried out in 30 µl instead of 15 µl previously used. Volume of DNA was limited to 1 µl.

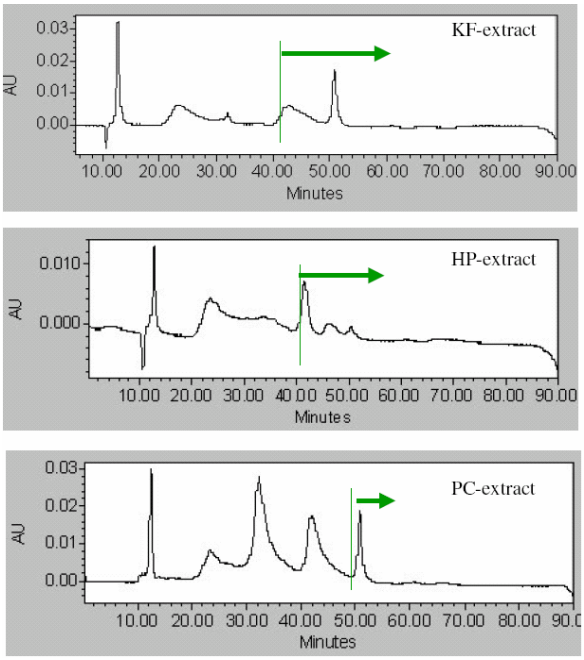


Figure 13: Chromatography of 4,65 µg KF-extract (top), 2,2 µg HP-extract (middle) and 6,4 µg PC-extract (bottom) of soybean meal on a DEAE 8HR column. Step gradient from 20% B (200 mM KCl) to 100% B (1 M KCl) after 55 minutes. Intermediate steps were set at 45% (15 min), 50% (25 min), 55% (35 min) and 60% (45 min). Arrows mark the appearance of PCR-positive fractions.

Secondly, the chromatograms in figure 13 show a considerable amount of UV-absorbing PCR-negative products, especially for PC-extracts. Actually none of these extraction protocols included an RNase digestion step. Digesting RNA in PC-extracts resulted in a different chromatogram (**figure 14**); none of the peaks between Rt 40 and Rt 55 remained visible, except for a small peak at Rt 51. PCR on the other hand showed positives between Rt 44 and Rt 55 min.

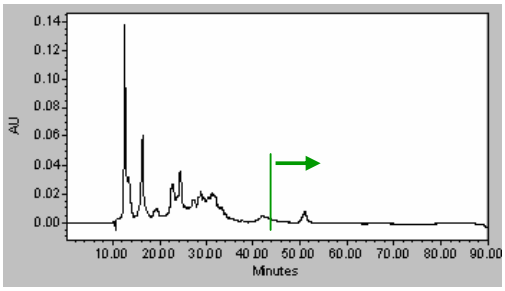


Figure 14: Chromatography of 6,4 µg PC-extract DEAE 8 HR column after RNase digest with 0.5 µg RNase for 10 minutes.

These results clearly indicate that a lot of RNA is co-extracted with DNA no matter which extraction protocol is applied and that the DNA amount in these extracts might be far lower than expected from OD absorbance measurements. Consequently, the calculated DNA amounts loaded on these columns were largely overestimated.
Figure 15 B shows UV-spectra of 2 fractions after plasmid DNA chromatography (DEAE 8 HR). The UV-spectrum of the second peak (Rt 50,8) is typical for nucleic acids.

On the other hand, no conclusions on purity can be drawn from UV spectra of KF-peaks (figure 13) as total absorption is too low (figure 15 B).

4.A.

4.B

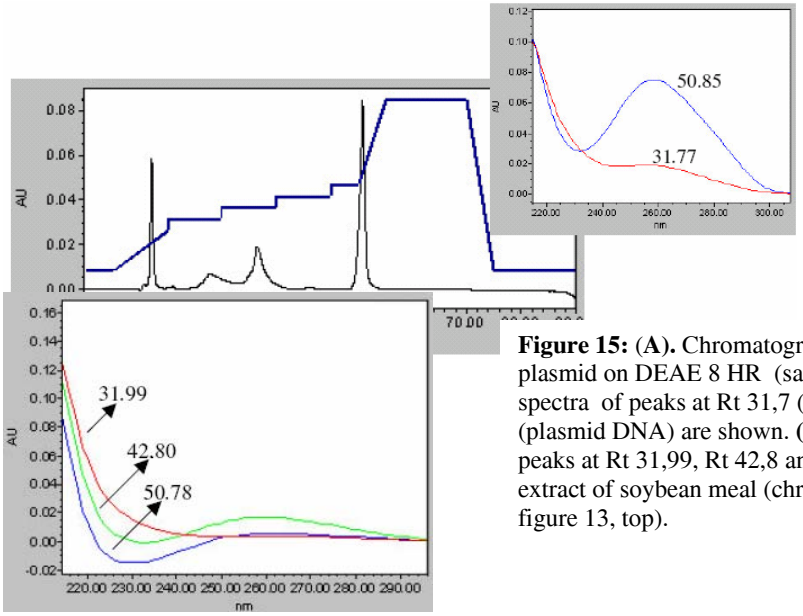


Figure 15: (**A).** Chromatography of 6 µg pLec2 plasmid on DEAE 8 HR (salt step gradient). UV-spectra of peaks at Rt 31,7 (RNA) and RT 50,8 (plasmid DNA) are shown. **(B).** UV spectra of peaks at Rt 31,99, Rt 42,8 and Rt 50,78 from KF-extract of soybean meal (chromatogram shown in figure 13, top).

Similarly, the OD260/280 ratios, considered to be an indicator of DNA quality, should be interpreted with care. Theoretically, pure DNA extracts have a ratio of 1,8 to 2,0. **Table 10** shows OD 260/280 ratios of HPLC peaks after genomic DNA chromatography and plasmid extracts shown in figures 13 and 15. Whereas ratios higher than 2.0 or below 1.8 can be indicative of the presence of impurities, values between 1.8 and 2.0 are not necessarily related to pure DNA. As an example, the OD 260/280 ratio for the KF-peak at Rt 42,8 is 1,97 but this peak is contaminated by RNA (shown by RNAse digest experiments).

	Extracts		
Rt (min)	pLec2	PC	KF
31,7	2,10		
50,85	1,91		
32,25		2,08	
42,10		2,11	
50,85		2,13	
42,8			1,97
50,78			2,12

Table 10: OD 260/280 nm ratios of genomic extracts and plasmid

A third observation is related to the retention times (Rt) of DNA. Rt of DNA extracted with phenol/chloroform (Rt 50) is higher, compared to DNA extracted with KF or HP extraction kits (RT 40 and 41, respectively). A possible hypothesis could be that the mean DNA fragment size of a PC-extract might be longer than that of HP- or KF-extracts and eluted later. Concluding, we can assess that although valuable information was present in chromatograms, no efficient purification of DNA was possible. Purification could be improved by using a step gradient instead of a linear gradient (**figure 16**).

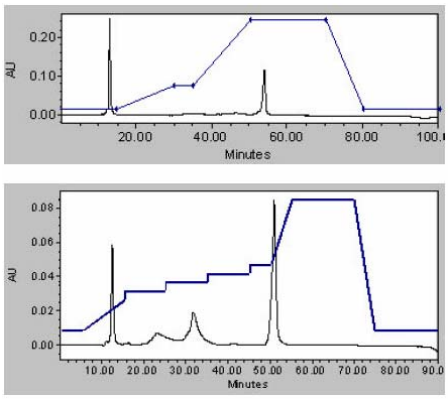


Figure 16: Chromatography of 5 µg pLec2 plasmid with linear gradient (top) and 6 µg pLec 2 plasmid with step gradient (bottom) on a DEAE 8 HR column. Whereas the linear gradient eluted plasmid extract as one peak (except from first elution peak), a step gradient separated RNA from plasmid DNA.

In an attempt to further improve separation of DNA from contaminating material we used two high resolution columns.

2.3.2.3.2 Gen Pak Fax (Waters) and DNA PAC PA-100 (Dionex)

The Gen PAK Fax and DNA PAC PA-100 are two high resolution columns differing by the nature of the packing material. The Gen PAk Fax is a weak anion exchange column, separating in a small pH range. It is packed with 2,5-µm particles essentially composed of DEAE functionalized non-porous, polymethacrylate resin. The DNA PAC PA-100 column is packed with 13-µm diameter non porous beads covered by 100-nm Microbead^{TM} alkyl quaternary ammonium functionalized groups.

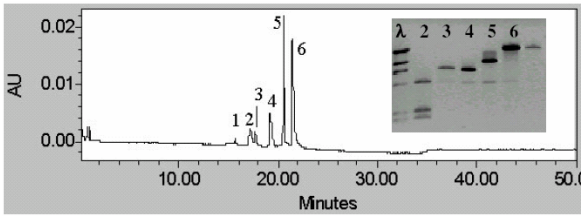


Figure 17: Chromatography of 2,5 µg λ/HindIII digest on Gen Pak Fax column (step gradient). Fractions were concentrated on centricon filters before gel electrophoresis (insert).

Figure 17 shows the chromatogram of 2,5 µg /HindIII digest on Gen Pak Fax using a step gradient. Six different peaks were visible. These peaks were analysed by gel electrophoresis subsequent to ultrafiltration on Centricon 30 kd (Millipore). Peak 1 was not collected but is assumed to contain the 525-bp fragment, peak 2 and 3 (collected in the same fraction) showed fragments 2027/ 2322 bp and 4361 bp, respectively; peak 4 contained the 6557-bp fragment, peak 5 fragment 9416 bp and peak 6 the 23120 bp-fragment. A similar high resolution was obtained with the DNA PAC PA-100, as injection of an uncut pLec 2 plasmid resulted in separation of the relaxed configuration form from the supercoiled one (figure 18). Gel electrophoresis was carried out after DNA concentration by inversed dialysis on a saccharose bed.

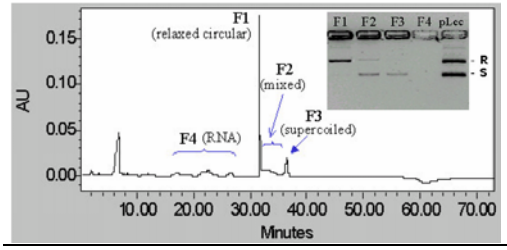


Figure 18: Chromatography of 5 µg non-digested pLec2 plasmid on a DNA PAC PA-100 (step gradient). Fractions 1 to 4 were concentrated by dialysis on a saccharose bed before agarose gel electrophoresis (insert)

Chromatography of the PC-extract on DNA PAC PA-100 with an improved step gradient showed that DNA eluted in a short lapse of time, although it still co-eluted with RNA. The application of a 0-375 mM perchlorate (NaClO4) instead of a chloride (KCl) gradient further improved separation of DNA, that eluted in a small peak adjacent to the last RNA peak (not shown)

Figure 19 shows chromatograms of PC-extracts on DNA PAC PA-100 after DNase and RNase digests. It shows that RNase digest completely flattens the UV-trace after Rt 20, whereas DNase digest hardly changed the chromatogram.

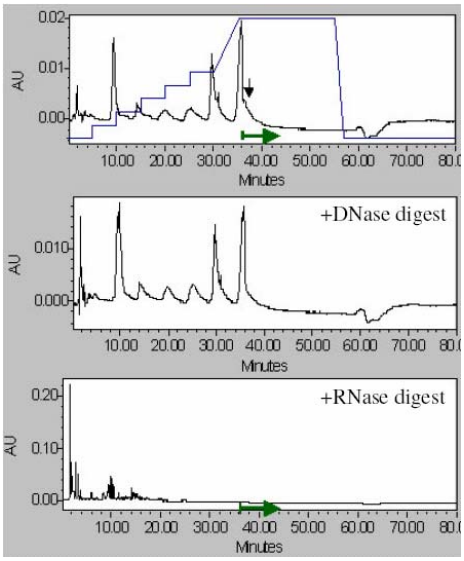


Figure 19: Chromatography of 6,4 µg PC-extract from soybean meal on DNA Pac PA 100 without enzymatic digest (top), with DNase digest (middle) and with RNase digest (bottom). DNase digest was carried out with 150 units DNase for 40 minutes at 37°C. RNase digest was carried out with 0,25 µg RNase for 30 minutes at 37°C (KCl step gradient).

These observations confirm that very large amounts of RNA were present in the extracts whereas DNA content was low. Similar conclusions could be drawn for HP- and KF-extracts although contaminating RNA was less abundant.

Because of RNA contamination in our PC-, KF- and HP-extracts, we decided to adapt our extraction protocols before proceeding to the third task of our project. First, RNase was added to the SDS lysis buffer prior to proteinase K digestion in the phenol/chloroform extraction procedure. Second, the thermolife extraction kit was replaced by the ***Wizard® Magnetic DNA Purification System for Food from*** Promega, which can also be combined to the King Fisher robot and includes an RNase digest step. The High Pure extraction protocol was discarded from our study, as this kit is not used for routine analysis of GMO's

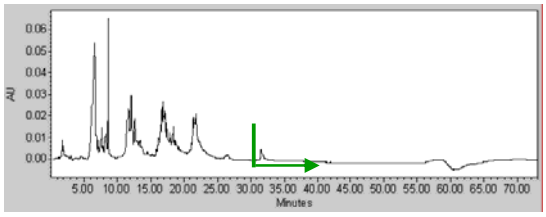


Figure 20: Chromatogram of 8 µg PC-extract treated with RNase during extraction.
$NaClO_4$ and step gradient were used. PCR positive fractions appear at Rt 31.

Figure 20 shows a chromatogram of a PC-extract after RNase digestion. Complete separation of DNA from contaminating molecules was achieved although a high amount of co-extracted molecules between Rt 5 and Rt 27 were still visible. This was in contrast with a chromatogram obtained on a PC-extract treated with RNase prior to injection. In the latter case, all residual RNA eluted before Rt 15. These results indicate that a lot of RNA is only

partially degraded when RNase digest is carried out during lysis step, probably because SDS impairs RNase activity.
Real time PCR was carried out on PC-extracts treated with or without RNase during the extraction procedure. Surprisingly, digested extracts showed a significant increase of amplification efficiency as values increased from 1,83 to 1,91. More striking is the reduction of variability, from 0,78 to 0,41. This is unexpected, first, because RNA is not considered to be inhibitory to PCR and, second, because the amount of RNA still present in our DNA extract was considerable. These results indicate that either large RNA molecules or high amounts of RNA might impair efficient DNA amplification. This could be due to low-stringent interactions between DNA and RNA in such way that DNA polymerase cannot target DNA properly.
Because of these high amounts of RNA remaining in most of the extracts, DNA quantification through absorbance measurements at 260 nm was still unreliable. Therefore different DNA quantification methods will be evaluated in chapter 4.

2.3.2.4 Characterization of DNA extracts from plant and feed matrices

Further characterization of DNA extracts were carried out with the Gen Pak Fax column as it had a higher capacity with equal resolution. For this purpose, twelve different matrices of plants or complex feed products were chosen in agreement with partner #5. Most of them were considered as problematic either because their extraction yields were low or the extracted DNA was of poor quality. Each matrix was extracted according to three extraction procedures: the phenol/chloroform extraction method (with RNAse digest) giving the highest DNA yields, the CTAB extraction procedure widely used to extract pure DNA from plant material and the Promega for food (PF) extraction kit that can be used in combination with the King-Fisher extraction robot.
Table 11 summarizes DNA concentrations by spectrophotometric measurements as well as DNA yields. CTAB and PC extractions were carried out on 1 gr of material and precipitated DNA was resuspended in 1 ml of water; the amounts extracted by the PFF kit are limited to 100 mg; final elution volume is 200 µl.

Reference	Matrix description	DNA extraction procedure					
		PC		CTAB		PFF	
		conc[a]	yield[b]	conc	yield	conc	yield
Raw material							
ext/2003/1	Maize kernels	231,5	0,23	36,63	0,036	21,9	0,044
ext/2003/2	Maize leaves	194,4	0,19	15,18	0,015	18,8	0,038
ext/2003/3	Fresh tomatoes	57,6	0,58	0,25	0,0025	8,2	0,016
Processed soybean products							
ext/2003/4	Soybean oil-cake	1737,7	1,74	17,7	0,017	12,1	0,024
ext/2003/5	Soybean proteins	3330,3	3,33	9,63	0,0096	12,7	0,025
Processed maize products							
ext/2003/6	Maize starch	11,3	0,011	1,55	0,0015	34,2	0,068
ext/2003/7	Maize gluten feed (I)	273,9	0,27	5,08	0,0051	6,75	0,013
ext/2003/8	Ensiled maize kernels	60,03	0,06	2,05	0,002	6,6	0,013
ext/2003/9	Maize gluten feed (II)	45,00	0,04	3,90	0,0039	6,8	0,014
ext/2003/10	Maize flakes	188,5	0,19	4,30	0,0043	8,5	0,017
Complex matrices							
ext/2003/11	Compound feed (I)	972,9	0,97	31,85	0,032	36,5	0,073
ext/2003/12	Compound feed (II)	1022,7	1,02	13,33	0,013	56,8	0,114

Table 11: DNA concentrations and yields of twelve plant and feed matrices

[a] DNA concentration expressed as µg/ml
[b] DNA yield expressed as µg DNA/mg of matrix. Phenol/chloroform and CTAB extractions: 1 gr of material for 1-ml DNA suspension. PFF extractions: 100 mg material for 200 µl DNA suspension

2.3.2.4.1 Inhibition of PCR by raw DNA extracts

DNA amplification on all extracts was carried out by conventional (partner #4) and real-time PCR (partner #5). Amplification by classic PCR was carried out on undiluted DNA extracts targeting a DNA sequence present in all extracts (universal primer) or a sequence selective to one species (species-specific primer). Real–time PCR was carried out as follows: for each extract, three dilutions were run in real-time PCR by targeting either a DNA sequence present in all considered extracts or a plasmid-borne target that was spiked in constant amount to each appropriate well. Absence of this last target in the several tested samples before spiking was checked.

Table 12: Inhibition of twelve plant and feed DNA extracts by real-time and classical PCR

	Extraction method →	PC				CTAB				PFF			
	Target →	Universal plant DNA		Spiked Mon810 Plasmid	Plant Species DNA	Universal plant DNA		Spiked Mon810 Plasmid	Plant Species DNA	Universal plant DNA		Spiked Mon810 Plasmid	Plant Species DNA
#		g5	g4	g5	g4	g5	g4	g5	g4	g5	g4	g5	g4
1	Maize kernels	I	I	I	I	I	N	N	N	N	N	N	N
2	Maize leaves	N	I	N	I	N	N	N	N	I	I	N	N
3	Fresh tomato	N	N	N	n.d	N	N	N	n.d	I	N	N	n.d
4	Soybean oil-cake	I	I	I	I	I	N	N	N	I	N	N	N
5	Soybean proteins	I	I	I	I	N	N	N	N	I	N	N	N
6	Maize starch	I	N[a]	I	N	N	N	N	N	I	I	I	I
7	Maize glutenfeed (I)	I	I	I	I	I	N	N	N	N	N	N	N
8	Ensiled maize kernels	N	N	N	I	I	N	N	I	N	N	N	I
9	Maize glutenfeed (II)	I	N[a]	I	N	N	N	N	N	N	N	N	N
10	Maize flakes	N	N	I	N	N	N	N	N	N	N	N	I
11	Compound feed (I)	I	I	I	I	I	N	N	N	I	N	N	N
12	Compound feed (II)	I	I	I	I	I	N	N	N	I	N	N	I

g4 = Group 4 (VAR) PCR results
g5 = Group 5 (CRA-W) real time PCR results
N=No inhibition effect
I = Inhibition of type I or type II (with visible extract dilution effect)
n.d. not determined
[a] Inhibition is observed when at least 2 µl DNA is added to a 15 µl-instead of a 30-µl reaction mix

Table 12 gives an overview of the inhibitory effects observed on classical and real time PCR. PC-extracts were most inhibitory to PCR whether universal or species-specific primers are used. In general, calculated DNA yields in these extracts were higher compared to CTAB- and PFF-extracts (table 11) but an increased yield of extracted DNA seems concomitant to an increased amount of co-extracted inhibitors. The CTAB procedure is reported to be very efficient for DNA extraction from plant material. Our results showed good efficiency on feed matrices too, whether processed maize, processed soy products or compound feed products were used. No inhibition is observed on CTAB-extracts using classic PCR. According to the supplier, PFF-extracts should give good quality DNA in amounts high enough to allow GMO detection. Still, complex matrices such as compound feed seem not to be suited for DNA analysis after PFF extraction as both PFF-11 and PFF-12 extracts show inhibition of PCR when species-specific primers were used. Similar experiments carried out by real time PCR give some unexpected results as detailed in the next paragraph

The first observation for results obtained by real time PCR is that inhibition may be absent in samples with high DNA content while it might be present in samples with a low total DNA content, especially if used undiluted. (see paragraph 2.3.3.2 for more detailed results on different types of inhibition observed by real time PCR).

2.3.2.4.2 HPLC analysis of relevant plant- and feed extracts.

In response to inhibition results obtained in the previous paragraph, some DNA extracts were studied in more detail by HPLC analysis that was largely commented in our previous report [27]. The next paragraph gives a summary of observations that were made.
First, as observed for soybean extracts in paragraph 2.3.2.3 of this report, DNA concentrations measured by absorbance at 260 nm did not correlate with estimations by other methods. The PC-extract from maize gluten feed I contains 54 times more DNA according to spectrophotometric measurements in comparison with the CTAB extract. This ratio is reduced to 2 when DNA peak areas were compared after HPLC. It should be noted that chromatograms of this extract showed a large amount of non-retained compounds resulting in a huge peak (AU 260: 0.295, **figure 21**) whereas the maximum absorbance of the DNA peak did not exceed 0.006 AU (**figure 21**, insert) Such contamination is expected to seriously bias DNA quantification by absorbance at 260 nm.

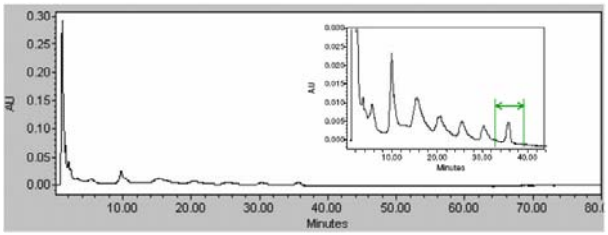


Figure 21: Chromatography of 50 µl PC/2003/7 extract from gluten feed I on Gen Pak Fax (step gradient). Insert shows enlarged view of chromatogram. Double arrow set bounds of PCR positive fractions.

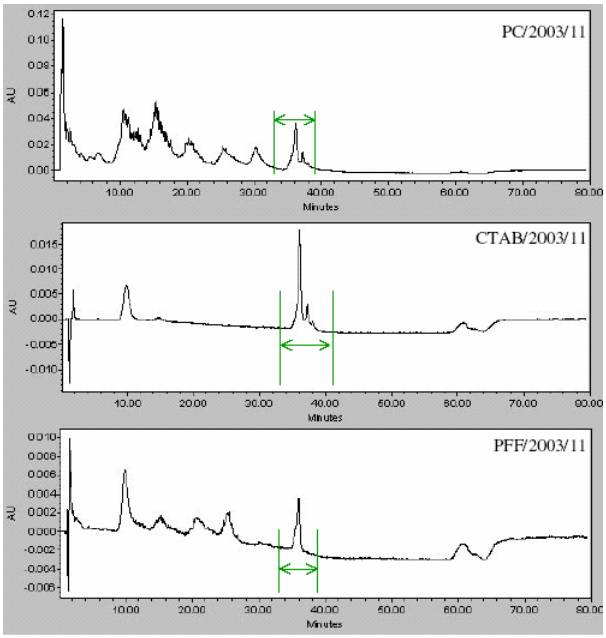


Figure 22: Chromatography on Gen Pak Fax of DNA extracts from compound feed I. From top to bottom 26 µl PC/2003/11, 51 µl CTAB/2003/11 and 50 µl PFF/2003/11 extracts are shown. Double arrows set bounds for PCR-positive fractions

Secondly, in all cases the amount of PCR-negative material after chromatography is more abundant in PC-extracts compared to CTAB or PFF-extracts (**figure 22**). A lot of this material

corresponded to RNA, despite the RNase treatment carried out during extraction. Furthermore, according to PCR results shown in table 12, PC-extracts were more inhibitory to PCR than other extracts.
Finally, PFF extracts from compound feed are not as pure as previously assumed and the ratio of extracted DNA against co-extracted compounds is hardly better compared to PC-extracts. CTAB extracts, on the other hand, were shown to be virtually devoided of any contaminating material. Actually, this protocol proved to be very efficient to eliminate RNA during DNA purification.
Concluding, DNA extracted by the phenol chloroform method from plant or feed matrices and more particularly from compound feed are strongly inhibitory to PCR, which seems to corroborate with high amounts of co-extracted material eluting early from anion exchange columns. We therefore decided to focus more specifically on these extracts for further characterization together with the gluten feed (I) extract, that is not only inhibitory to PCR, but is particularly contaminated by co-extracted compounds.

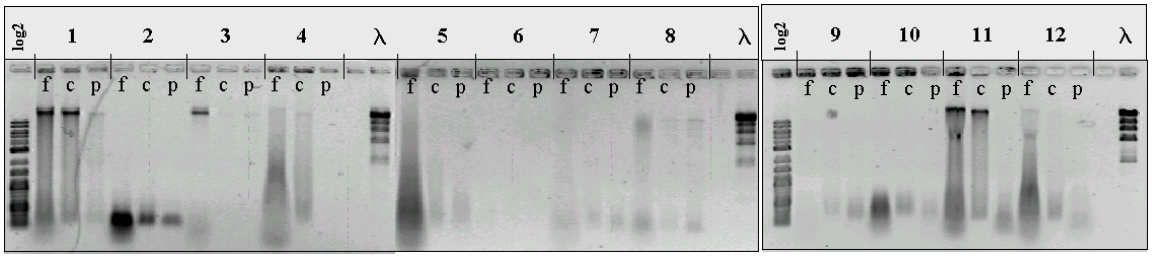
2.3.2.5 Characterisation of DNA contamination

2.3.2.5.1 Quantification of DNA from plant and feed extracts

Gel electrophoresis analysis

As mentioned before, quantification of DNA proved to be much harder than generally accepted. Theoretically, agarose gel electrophoresis allows to compare DNA amounts between different extracts but practically DNA degradation or precipitation during gel loading might prevent correct interpretation.

Figure 23: Gel electrophoresis on DNA extracts from twelve plant and feed matrices. Extractions were carried



out according to three procedures: 10 µl of phenol/chloroform (f) and CTAB extracts (c) and 20 µl of PFF extracts (p) were loaded on the gel. Numbers refer to type of matrix. **1**. maize kernels; **2**. maize leaves; **3**. fresh tomatoes; **4**. Soybean oil-cake; **5**. soybean proteins; **6**. maize starch; **7**. maize gluten feed I; **8**. ensiled maize kernels; **9**. maize gluten feed II; **10**. maize flakes; **11**. compound feed I; **12**. compound feed II.
Abbreviations: Log2 (Biolabs) is a DNA ladder with a maximum band size of 10 kb; λ = Lambda phage/HindIII digest (Invitrogen).

Figure 23 is an electrophoregram of DNA samples extracted from all 12 matrices according to three different extraction protocols mentioned earlier. Only few extracts show a distinctive DNA band around 23 kb.
Although quantification by spectrophotometry is largely overestimating the DNA amount, gel electrophoresis confirmed that extraction by the phenol/chloroform procedure yielded the highest DNA amounts. However, the DNA purity is questionable, especially for extracts from maize kernels or complex feed products, which showed strong inhibition. On the other hand,

some matrices could be extracted with good efficiency such as fresh tomatoes (extract 3) for which no inhibition was observed, neither by classic PCR, nor by real-time PCR.
According to table 11, CTAB extracts contain low amounts of DNA. Nonetheless, DNA concentrations of PC- and CTAB-extracts might not be as different as initially presumed. Indeed, for some samples, the intensity of DNA-bands of PC- and CTAB-extracts (more particularly extracts 1 and 11) was not as different as measured spectrophotometrically (972,9 µg/ml for PC/2003/11 against 31,85 for CTAB/2003/11). Similar observations were made when areas under DNA peaks after anion exchange chromatography were compared to absorbance measurements.
Finally, as judged by gel electrophoresis, PFF extractions yielded the lowest amount of DNA and no DNA band could be detected in "Promega for Food kit"-extracts. This was contrary to results obtained by spectrophotometry (see table 11). In the latter case, calculated concentrations and DNA yields were actually higher compared to CTAB extracts. One of the main reasons quantification of PFF extracts is overestimated is turbidity. Bias could be reduced by centrifugating extracts prior to OD measurements or by withdrawing OD value measured at 320 nm from that obtained at 260 nm (preceded or not by DNA denaturation in NaOH 2M). **Table 13A** shows results of PFF extracts that were quantified spectrophotometrically without denaturation (OD_{260}-OD_{320}), after extract centrifugation or by combining both methods. Concentrations of processed maize showed largest differences, maybe because co-extracted starch has not completely solubilized. Consequently, resulting turbidity interferes with absorbance measurements.

A

Spectrophotometric quantification of PFF extracts expressed as µg.ml^{-1}					
#	Matrix	OD_{260nm}	$OD_{260nm - 320nm}$	Centrifugation/ OD_{260nm}	Centrifugation/ $OD_{260nm-320nm}$
Raw products					
1	Maize kernels	26,8	21,1	21,9	21,0
2	Maize leaves	19,4	17,5	18,8	17,6
3	Tomatoes	16,6	6,25	8,2	6,15
Processed soybean products					
4	Oil-cake	12,1	11,85	12,1	12,1
5	Proteins	10,6	10,55	12,7	12,35
Processed maize products					
6	Starch	53	29,85	34,2	31,5
7	Gluten feed (I)	37,3	11	6,75	6,1
8	Ensiled kernels	43,2	10,22	6,6	5,2
9	Gluten feed (II)	20,2	9,65	6,8	6,0
10	Flakes	110,6	16,12	8,5	7,75
Complex feed products					
11	Compound I	80,6	44,4	36,5	32,7
12	Compound II	106,3	54,8	56,8	48,9

B

Spectrophotometric quantification of DNA (µg.ml^{-1})				
#	Matrix	Centrifugation/OD_{260nm}-OD_{320nm}		
		PC	CTAB	PFF
Raw Products				
1	Maize kernels	211,5	30,2	20,75
2	Maize leaves	153,5	15,75	17,6
3	Fresh tomatoes	56,0	0,8	6,15
Processed soybean products				
4	Oil-cake	1682	14,95	12,1
5	Proteins	3548	8,8	12,7
Processed maize products				
6	Starch	1,5	1,5	31,5
7	Gluten feed (I)	39,25	3,75	6,1
8	Ensiled kernels	31,75	3,1	5,2
9	Gluten feed (II)	17	2,95	6,05
10	Flakes	128,75	4,45	7,75
Complex feed products				
11	Compound I	567	18,9	32,1
12	Compound II	402,5	11,75	48,9

Tables 13: (A) Spectrophotometric quantification of PFF extracts. **(B)**: Quantification of PC-, CTAB- and PFF-extracts

Overall, centrifuging extracts prior to quantification resulted in the lowest bias on calculated concentrations. It largely abolished OD_{320} (concentrations in two last columns are similar) and

therefore it seemed the most appropriate method to quantify DNA-extracts spectrophotometrically. PC- and CTAB-extracts were quantified again after centrifuging all extracts. Results were striking especially for maize processed products such as starch, for which calculated concentration was reduced 7-fold from 11,5 µg/ml down to 1,6 µg/ml. As opposed to the determination of PFF-concentrations some PC-extracts (DNA extract from gluten feed I) showed significant absorbance at 320 nm after centrifugation. We therefore combined both centrifugation and OD_{320} withdrawal for determination of DNA concentrations (**table 13B**). Overall, concentration ratios between PC- and CTAB-extracts remain high.

Evaluation of different quantification methods

We selected 2 matrices from which genomic DNA was extracted according to three previously mentioned protocols, one commercial genomic DNA extract (Calf Thymus DNA, Sigma) and a plasmid DNA (pLec2). All samples were quantified by 5 different methods: Absorbance at 260 nm (with or without denaturation of dsDNA by NaOH 2M), picogreen fluorescence, dot densitometry, estimation of DNA peak areas and real-time PCR (**table 14**).

Quantification of genomic and plasmid DNA (µg.ml^{-1})						
	OD dsDNA[a]	OD ssDNA	Picogreen	Densitometry	DNApeak[b]	Q_PCR[c]
CT-DNA	1400	951	570[e]	1210	2562	/
pLec2[d]	0,64	/	0,34	0,497	494	/
PC/2003/11	567	517,56	18,65	118,5	100	100
CTAB/2003/11	18,20	16,87	7,25	15,3	31,9	25,62
PFF/2003/11	32,1	31,35	0,31	/	8	3,19
PC/2003/12	405	436,24	7,1	60,9	84,8	54,24
CTAB/2003/12	11,75	9,20	1,45	5,5	9,4	9,76
PFF/2003/12[d]	48,9	41,23	0,10	/	6,4	1,83

Table 14: Comparison of different quantification methods

[a] OD measurements of feed extracts were taken after centrifugation at 12000 rpm for 10 min.
[b] peak areas are given for 50 µl undiluted sample
[c] Q-PCR : quantitative or real-time PCR. Results are given in percentages. 100% = amplification result of PC/2003/11 extract
d plasmid was shown to contain RNA, densitometry was carried out after gel electrophoresis
e result is derived from 50-fold dilution of stock concentration.

The first column shows spectrophotometric results of DNA extracts. All feed extracts were centrifuged prior to $OD_{260nm-320nm}$ measurements but results remained too high and unreliable for quantification of genomic DNA at least when dealing with impure extracts. The second column shows OD_{260nm} results of denatured DNA extracts after $OD_{320\ nm}$ correction. Concentrations are very similar to the previous column or to results obtained without denaturation (compare results for PFF extracts with those in table 15). Consequently, denaturation of DNA prior to quantification might not be useful.
Picogreen fluorescence (third column) is reported as a good alternative to absorbance measurements but we showed that this method is very sensible to DNA dilutions. As a result, quantification of diluted DNA extracts are underestimated. For instance, CT-DNA could be quantified with reasonable reliability when diluted 50 times. At a 140-fold dilution, fluorescence dropped 3-fold resulting in a calculated concentration of 200µg/ml instead of 570 µg/ml. Similar observations were made for PC- or CTAB extracts and PFF extracts were not quantifiable, probably because initial DNA concentrations were too low or too diluted. Picogreen measurements were done by Dr. Corbisier (IRMM, Geel) and partner #5.

Dot densitometry gave interesting results as concentration values of PC extracts lay between OD and picogreen results. Preliminary results for CTAB extracts showed good correlation with OD results. However, in general, this method required too much material, which makes it unsuitable for quantification of low amounts of DNA such as PFF and some CTAB extracts and it revealed to be too difficult to reproduce in the long term to be useful for routine DNA quantification. HPLC analysis was suitable for comparison of genomic DNA extracts but no standard genomic DNA was found for quantification of these extracts.
Real-time PCR is target-specific and proportional to genomic DNA content. Absolute quantifications is therefore very difficult.

Concluding, quantification is only possible when dealing with truly pure DNA such as plasmids recovered from HPLC or CsCl-gradients. In these cases, quantification can be carried out according to all methods and more specifically by spectrophotometry, densitometry or picogreen fluorescence.
Quantification of genomic DNA proved to be very hazardous. Quantification variability of commercial calf thymus DNA reached up to 60% between different quantification methods if DNA extracts were not too much diluted.
Quantification of genomic DNA extracts from plant or feed extracts is even more unreliable. Even though CTAB extracts were shown to be pure, different quantification techniques gave large variations probably because of undetected interfering compounds.
Therefore, as for plasmid DNA, genomic DNA can only be quantified with reasonable accurateness if dealing with a pure extract, which none of our extraction methods is able to achieve. Some consensus should be defined for DNA quantification, keeping in mind it will not be a true estimation of the absolute DNA amounts.

2.3.2.5.2 Analysis of co-extracted compounds in DNA extracts from plant and feed

Only matrices 7, 11 and 12 were selected for determination of inhibitors. PC-, CTAB- and PFF-extracts were separated on disposable anion-exchange columns. Sep-Pak® light Accell™ Plus QMA columns (Waters) were used and early eluting fractions were collected for analysis of inhibitors.

Purification of DNA extracts 7, 11 and 12 was very efficient as 100 µl raw extract could be applied on these columns. DNA was only released after elution with 100 % buffer B containing 1 M KCl. The eluted DNA fraction was pure as verified by HPLC analysis (**figure 24**).

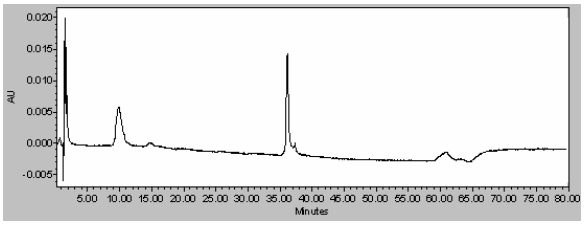


Figure 24: Chromatography of 50 µl PC/2003/11 extract on Gen Pak Fax after purification on a SepPak column. The purified fraction was dialyzed against a desalted buffer in a Spectra/Por membrane MWCO 6-8 kd (Spectrum Laboratories) and concentrated on a sucrose bed prior to injection.

Buffers used for SepPak columns were identical to HPLC buffers. Three elution steps were carried out : 10%B (flow-through fraction), 30%B and 70%B. In the first step, the flow-through fraction was collected by passing 500 µl 10 % B buffer on the column. Three wash steps of 500 µl each were passed. The second and third step was carried out by passing 1200

µl buffer B at 30% and 70%, respectively and collecting the first 400 µl for analysis. In the final step 10 fractions of 100 µl buffer B 100% were collected. DNA eluted in the first 2 or 3 fractions.
All fractions were dialysed in a Slide-A-Lyzer cassette of 0.1 - 0.5 ml, MWCO 3,5 kd (Pierce) to eliminate KCl. Abu-al-Soud and co-workers [28] showed that Taq polymerase is inhibited by KCl at concentrations 60 mM above PCR buffer concentration (50 mM KCl).
Concomitant to DNA separation on SepPak columns, CTAB-extractions of 6 certified transgenic soybean and maize meals (IRMM, Geel Belgium) were carried out. These were RoundUp Ready soybean (RRS), Bt 176 maize and Bt11 maize at 0.1% and 1% transgenic material each. Amplificability was tested by PCR using serial dilution of DNA. Non-transgenic DNA could be amplified up to 1000- or 10000-fold dilutions. Transgenic DNA could at least be amplified up to 10-fold dilution (not shown)
To determine whether early eluting compounds from the SepPak column are inhibitory to DNA amplification, PCR assays were carried out with 2 µl serial-diluted reference DNA added to 3 µl SepPak fractions. Both universal plant primers and species-specific primers were used on maize and soybean DNA. **Table 15** summarizes results obtained for nine extracts and 2 negative controls (water and calf thymus

Table 15: Inhibition by co-extracted compounds of serially diluted standard DNA

		Maïze DNA				Soybean DNA				
	Primers	UP1-ITS2		Zein3-Zein4		UP1-ITS2			SI9-SI10	
	DNA dilution	-1	-2	-1	-2	-1	-2	-3	-1	-2
H_2O	Flow-through (FT)	/	+	+	+	/	+	+	+	+
	30% buffer B (30B)	+	+	+	+	+	+	+	+	+
PC-extract	PC12-FT	-	-	+	-	-	-	-	-	-
	PC12-30B	-	-	-	-	-	-	-	-	-
	PC11-FT	-	-	+	+/-	/	+	-	/	+
	PC11-30B	-	-	-	-	-	-	-	-	-
	PC7-FT	-	-	+	-	+	-	-	+	-
	PC7-30B	+	-	+/-	-	-	-	-	-	-
CTAB-extract	CTAB12-FT	/	+	+	+	/	+	+/-	/	+
	CTAB12-30B	+	-	+	-	/	+	-	+	-
	CTAB11-FT	/	+	+	+	/	+	+/-	/	+
	CTAB11-30B	+	-	+	+/-	/	+	-	/	+
	CTAB7-FT	/	+	+	+	/	+	+/-	/	+
	CTAB7-30B	/	+	+	+	/	+	+	/	+
PFF-extract	PFF12-FT	/	+/-	+	+	/	+	+	/	+
	PFF12-30B	+	-	+	-	+	-	-	+	-
	PFF11-FT	+	-	+	-	+	-	-	+	-
	PFF11-30B	+	-	+	-	+	-	-	+	-
	PFF7-FT	+	-	+	+	+	-	-	+	+
	PFF7-30B	+	+	+	+	/	+	-	+	+
CTD[1]	CtDNA-FT	/	+	+	+	/	+	-	/	+
	CtDNA-30B	+	+	+	+	+	+	+	+	+

[1]CTD stands for Calf thymus DNA
PCR results carried out in duplicate: **+** both positive; **-** both negative; +/- one positive, one negative;
/ not determined

Results revealed that besides water and calf thymus, only CTAB-extracts from gluten feed I (CTAB-7) showed no inhibition at all conditions tested. In contrast, 30B-fractions of PC-11 and PC-12 extracts showed inhibition in all conditions. Despite their apparent purity CTAB-

11 and CTAB-12 fractions were inhibitory to amplification of 100-fold diluted standard DNA. Previously, real-time PCR measurements already revealed that CTAB-11 and CTAB-12 were inhibited upon amplification of a universal plant primer (table 12).
Finally, PFF extracts showed that SepPak fractions from compound feed extracts are much more inhibitory to PCR compared to CTAB extracts. Therefore, PFF extraction kits might not be suited for quantification of DNA from complex matrices such as compound feed.
Overall, compound feed I-extracts (ext-11) were less inhibitory to PCR compared to compound feed II-extracts (ext-12) and the FT-fractions (flow-through) were less inhibitory to PCR, compared to 30B-fractions. A possible explanation could be that unretained co-extracted compounds were composed of positively charged or neutral compounds such as neutral polysaccharides, which are not inhibitory to PCR [29]. Gluten feed I extracts contained a large amount of unretained co-extracted compounds as judged by a huge peak eluting in the first 2 or 3 minutes (figure 21). -amylase and amyloglucosidase digestions followed by glucose determination of this peak revealed that starch constituted a major compound (476 µg in pooled fractions from 1 injection), but its content was not significantly higher compared to flow-through fraction of PC-12 extract (441 µg). The exact nature of this peak remains unknown.
One of the reasons 30B-fractions were more inhibitory to PCR might be linked to the presence of acidic polysaccharides in these fractions. As these are negatively charged at basic pH, they are retained on the anion exchange column. Moreover, addition of ice-cold acetone to these fractions led to precipitation of the inhibitory compound, which correlates with behaviour of acidic polysaccharides. Actually, inhibition results of acetone-precipitated SepPak fractions were, with few exceptions, identical to those given in **table 16**. Only, CTAB 12-30B, CTAB 11-30B, PFF12-30B and PFF11-30B were not inhibitory to PCR after acetone precipitation in conditions for which inhibition was observed without acetone precipitation. Either inhibitory compounds were not precipitable or they were too diluted to have any effect. Some of these fractions were further investigated by adding facilitators to the PCR reaction in order to suppress inhibition of PCR (see paragraphe2.3.2.5.3). Supernatants after acetone precipitation never induced inhibition of PCR.

In a second series of experiments, DNA amplification of 0,1 % transgenic DNA was compared to 10-fold diluted 1% transgenic DNA. Practically, DNA extracts were quantified spectrophotometrically after DNA denaturation and similar amounts of transgenic DNA were used in the reaction. Amplification rates of 0.1 and 1% transgenic DNA were similar in the absence of SepPak fractions as judged by the gel electrophoregram of PCR products (not shown). On the other hand, when 3 µl SepPak fractions were added to the reaction, 0,1% transgenic DNA was amplified more efficiently compared to diluted 1% transgenic DNA. These results seem to indicate that PCR is less efficient on diluted extracts. Therefore, sample dilution might not always be a solution for suppression of PCR inhibition. Results for soybean DNA are summarized in **table16**.

Table 16: Inhibition by co-extracted compounds of RoundUp Ready soybean DNA

Inhibition by co-extracted compounds of RRs soybean DNA at 0,1% and 1 %								
Extraction procedure			PC		CTAB		PFF	
Transgenic content			0,1%	1%	0,1%	1%	0,1%	1%
Dilution of transgenic DNA			2x	9,58x	2x	9,58x	2x	9,58x
Primers			EPSPS1-EPSPS2					
SepPak fractions	H_2O	FT	++	++	++	++	++	++
		30B	++	+	++	+	++	++
	gluten feed (I)	FT	++	++	++	++	++	++
		30B	++	-	++	++	++	++
	compound feed (I)	FT	+	+/-	++	++	++	++
		30B	++	-	++	++	++	+
	compound feed (II)	FT	+	+/-	+/-	+/-	++	++
		30B	-	-	+	-	++	+/-

2.3.2.5.3 Suppression of PCR inhibition

Enhancing agents

Enhancing agents or facilitators have been frequently described to reverse inhibition of DNA amplification by PCR. Generally, inhibition is either linked to target DNA itself, to the Taq DNA polymerase or to a combination of both.
Enhancing agents are commonly used products that are, generally, selected by trial and error. Underlying mechanisms are poorly understood and often hypothetical. Practically they will only be effective if PCR inhibition is “weak” either because the inhibitor:DNA ratio is low or because interactions between inhibitor and target are weak.
Amongst frequently reported facilitators we have used BSA, T4 gene 32 protein (gp32), dimethyl sulfoxide (DMSO), glycerol, tween 20, PEG 400, formamide and magnesium (table 10)
BSA and gp32 may act as decoys for Taq DNA polymerase. They were both reported to increase the yield of PCR products amplified from samples containing humic, fulvic or tannic acids. They probably enhance PCR response by serving as targets for formation of hydrogen bonds between (poly-) phenolic compounds and peptide bonds [30, 31] or by binding proteinases. Alternatively, they share the ability to protect target DNA from unspecific entrapment by organic material [32]. BSA also binds to lipids via hydrophobic forces and is able to capture anions by its high lysine content. gp32 is a single-strand specific and thus helix-destabilizing protein encoded by gene 32 of the phage T4 genome. It might facilitate PCR by retarding reannealing as a result of binding to denatured single strand target DNA. DMSO as well as formamide are denaturants reducing base-pairing of GC-rich strands and consequently enhance primer annealing[33, 34]. PEG 400, together with DMSO and glycerol has been described to relieve inhibition from acidic polysaccharides.
Magnesium is not considered as an enhancing agent but increasing magnesium concentrations might compensate for Mg^{++}- depletion by complexing agents such as EDTA or humic acids.
Several agents that facilitate product formation in PCR amplifications are now commercially available as a mix.
Finally, we also tested RNase digest on SepPak fractions prior to PCR as we previously showed that RNA impairs efficient DNA amplification

Table 17

Enhancing agents	Concentration
BSA	4 mg/ml; 0.4 mg/ml
gp32	100 µg/ml
DMSO	10 %
glycerol	10 %
tween 20	0,5 %
formamide	1 %
magnesium	+ 1,5 mM
RNase	1 µg/µl
Solution Q	5-fold diluted

The ability of these agents to enhance DNA amplification in the presence of inhibiting SepPak fractions was tested. PCR was carried out on 100-fold diluted maize or soybean DNA in the presence of 3µl SepPak fractions. Enhancing agents were added at indicated concentrations (**table 17**). None of the agents could relieve inhibition by PC-11 or PC-12 SepPak fractions.
DMSO 10% was able to partially or totally relieve PCR inhibition of soybean DNA in the presence of PC7-FT and PFF12-30B fractions. Both DMSO 10% and solution Q were able to significantly improve amplification of soybean DNA in the presence of PFF 7-FT fraction. Composition of solution Q (Qiagen), is unknown but as most of these "ready-to-use" mixtures, it probably contains DMSO.
Only, inhibition by SepPak fraction CTAB12-30B could be relieved by more than one agent as shown in **figure 25**. Electrophoregram of PCR products from maize DNA shows that inhibition was relieved when DMSO 10%, glycerol 10%, gp32 0,1 µg/ml and solution Q was added to the reaction.

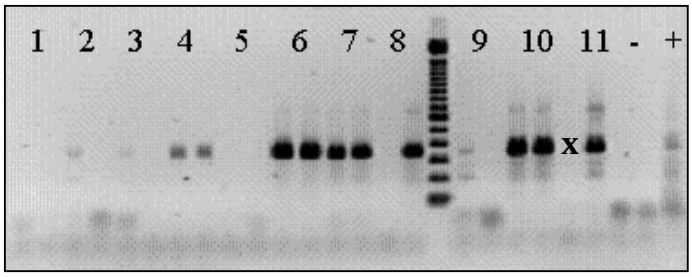


Figure 25: Electrophoregram of PCR products from maize DNA in the presence of CTAB12-30B fractions supplemented with following enhancing agents: **1.** none **2.** 4mg/ml BSA **3.** 0.4 mg/ml BSA **4.** 10% DMSO **5.** 0.5% Tween 20 **6.** 10% glycerol **7.** 0.1 mg/ml gp32 **8**. 1% formamide **9.** 1.5 mM $MgCl_2$ **10.** solution Q **11**. Rnase digest. Reactions are shown in duplicate except for lane 11

As opposed to gp32, only a slight band was visible when BSA was added to the PCR reaction. This indicates that gp32 acts through specific ssDNA binding properties rather than properties common to both proteins.
In our preliminary study, RNA was shown to impair DNA amplification when present in large amounts. We therefore carried out RNase digestion on SepPak fractions prior to PCR. As shown on figure 25, it allowed relieve of inhibition by the CTAB12-30B fraction. RNase digest also enhanced PCR response in the presence of CTAB11-30B (not shown). This is rather unexpected as CTAB extracts were shown to contain very little RNA. Digested PC-extracts remained inhibitory to PCR, probably because inhibition is caused by other contaminating agents such as acidic polysaccharides.
PEG 400 is frequently reported to enhance PCR response but, in our hands, PEG 400 was inhibitory to PCR. CTAB11-30B, CTAB12-30B and PFF12-30B induced no inhibition after acetone precipitation (see paragraph 2.3.2.5.2).

Adaptation of extraction protocol

The phenol-extraction procedure was initially used without RNase digestion. During this study, we realised RNase digest was essential to improve efficiency of real-time PCR. However, despite introduction of RNase digest, HPLC analysis revealed that a lot of partially digested RNA remained in the extract. Actually, RNase digest is carried out in the lysis buffer containing 1% SDS, which probably inhibits the enzyme and consequently reduces digest efficiency. We therefore decided to introduce RNase digest at the end of the extraction procedure. Practically, the extraction procedure is carried out without RNase digestion during lysis step and only one phenol/chloroform extraction step is carried out before precipitation of DNA with EtOH/acetate. DNA is subsequently resuspended in Tris buffer containing 50 µg/ml RNase and incubated at 37°C for 30 min. A second phenol/chloroform extraction is then carried out to eliminate RNase. DNA is precipitated again with EtOH/acetate and washed once in EtOH 70% before being resuspended in water.
Gel electrophoresis of DNA extracts from gluten feed I (extract 7) and compound feed I and II (extracts 11 and 12) is shown in **figure 26**. Phenol/chloroform extractions without RNase digest (a), with RNase digest during lysis (b) and with RNase digest at the end of procedure (c) are shown

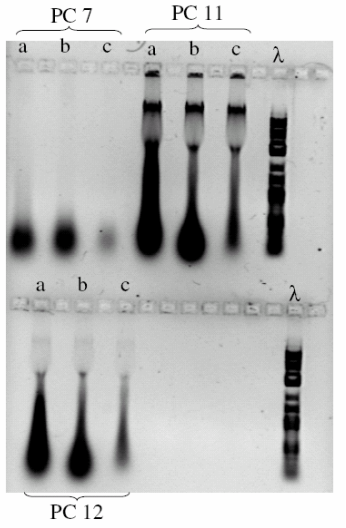


Figure 26: Gel electrophoresis of 10 µl DNA extracts from gluten feed I (PC 7), compound feed I (PC 11) and compound feed II (PC 12). Phenol/Chloroform extractions were carried out without RNase digest (a), with RNase digest during lysis step (b), with RNase digest at end of procedure (c). DNA molecular weight marker: lambda/HindIII digest (λ).

Whereas RNase digest during the lysis step had little effect on total RNA amount, the latter is drastically reduced when RNase digest is introduced at the end of extraction procedure.
PCR was carried out on all extracts either targeting a species-specific maize DNA or a spiked transgenic DNA extracted from a 1%- Bt 11, Bt 176 and RRS mixture.
No significant difference could be observed between different PC-7 extracts when species-specific targets are amplified. On the other hand, amplification of DNA from PC-7c extracts was significantly improved when these were spiked with 100-fold diluted transgenic DNA. It should be noted that amplification of transgenic soybean DNA is much more efficient as compared to transgenic maize DNA probably because extraction of the former is more efficient.
PCR results of PC-11 and PC-12 extracts are improved when RNase digest is carried out at the end of the procedure no matter whether a species-specific or transgenic DNA is targeted. Indeed, inhibition of 2-fold diluted “c”-extracts could be relieved.

2.3.2.6 Conclusion

To conclude with this topic, we would like to pinpoint that there is no simple method to define “chemical descriptors” for DNA quality, mainly because the nature of genomic DNA extracts is far too heterogeneous. HPLC analysis allowed successful separation of DNA from other contaminants and gave a rapid estimation of DNA purity. In many cases the amount of DNA truly present in the extract was far lower than initially presumed. However, the HPLC - method cannot be included in a purification protocol as it is complex and time consuming.
We have clearly shown that PCR failure can be explained by the presence of matrix-dependent contaminants that could be isolated by the use of anion-exchange cartridges. We also showed that the addition of an RNase digest-step in the phenol-chloroform extraction method should be a standard procedure and that purity of extracts is greatly improved when this step is carried at the end of the procedure. Quantification of genomic DNA remains a major problem, as there is no universal and reliable method. Actually, both total DNA quantification by spectrophotometry and target DNA quantification by real-time PCR constitutes no major problem as long as pure genomic DNA extracts are used. We therefore believe much more attention should be drawn to the purification of extracted DNA from feed products. Clean-up of complex DNA extracts by the use of well-known anion-exchange cartridges could be an option if sufficient DNA can be extracted.

	Advised protocol	Comments
maize kernels	CTAB or PFF	very little contamination observed by HPLC, almost no inhibition except for universal plant DNA by real-time PCR. PFF shows no inhibition but amount of extracted DNA is much lower as judged by gel electrophoresis. Absorbance measurements are comparable, though
maize leaves	CTAB	no visible contaminants by HPLC, no inhibition. PC is strongly contaminated by what we believe to be RNA that is hardly degraded. Strong bias on quantification. PFF produces similar amounts but inhibited by universal primer
fresh tomatoes	PC	DNA yield much higher as compared to CTAB or PFF extraction protocols correlated by very nice DNA band by electrophoresis, hardly any contaminating RNA visible on gel. No inhibition on PCR, nor by real –time PCR, nor classic PCR
soybean proteins	PFF or CTAB	PFF- and CTAB-yields are comparable as judged by HPLC and spectrophotometric quantification. Both extracts are readily pure 5HPLC). As CTAB is too laborious PFF seems advisable although some inhibition was found for PFF extract by real-time PCR on universal plant.. PC extracts are too much contaminated by co-extracted compounds and are strongly inhibited. Although total DNA amount is much higher as compared to CTAB or PFF, quantification is largely overestimated and extracts are strongly inhibited and therefore not suited for detection of DNA at low amounts
soybean oil-cake	CTAB	DNA yield is much higher as compared to soybean proteins but some inhibition on total universal plant target remains. No ideal solution but PFF and PC- do not seem to be advisable as both perform much worse as compared to soybean proteins and are inhibited too. PFF is totally inefficient for DNA extraction from oil-cake. despite spectrophotometric quantification equivalent to soybean proteins, HPLC shows DNA peak 10-fold smaller. Co-extracts contaminants seem to interfere with quantification
starch	CTAB	very low yields both for CTAB and PC but PC are inhibited. PFF extracts seem to yield higher amounts but very inhibitory. Not enough information available
gluten feed I	CTAB or PC	PC if RNase digests is carried out at end of protocol. Many interfering material for quantification but PCR response is OK
compound feed I and II	CTAB	although RNase digest carried out at end of procedure, there is still inhibition. moreover DNA yields are not significantly different between CTAB and PC extracts as judged by electrophoregram and HPLC analysis. In addition many contaminants are co-extracted

Table18: Summary of advised extraction method in regard of analyzed samples

2.3.3 Characterization of DNA extracts by real time PCR (partner #5).

Quantitation of GMO's (or any other target) by real time PCR requires an in depth knowledge of the kinetics of genetic amplification reactions. The aim of this part of the work is to characterize the quality of DNA extracts by means of their influence on PCR kinetics. In fact, one of the major problems in GMOs quantitation is the variable quality of extracted DNA. Damaged DNA molecules or residual impurities can interfere with DNA amplification. The three main traits that are assessed are the amplification efficiency, the possible inhibitory effect on PCR of an extract and the variability on quantitation by real time PCR. During this project, three interdependent approaches were used.

1. Analysis - curve by curve - of real time PCR kinetics and variability on quantitation on a determined copy number of DNA molecules prepared with different extraction methods from a model matrix (soybean).
2. Analysis of the PCR efficiency and the possible presence of PCR inhibitors in DNA extracts prepared from several matrices that are currently analysed in routine and of which some were known to have shown a very strong inhibitory effect.
3. Analysis of the capacity of DNA extracts to be amplified by real-time PCR by determination of detection and quantitation ranges on a target obtained from a model matrix by different extraction methods.

2.3.3.1 Analysis of real time PCR kinetics and variability on quantitation

2.3.3.1.1 Methodology

The aim of this point is to analyse the real time PCR kinetics -curve by curve- on a defined copy number of DNA molecules prepared with different methods from a model matrix focusing our attention on two parameters : the PCR efficiency and the variability on quantitation.

Choice of model matrix, DNA extraction methods and Standard DNA

Analyses were performed on soybean flour, taking advantage of our experience with this matrix [35]. The soybean was ground (with Retsh, Model ZM100 grinder) to have particles less than 500µm (this particle size fits with the AFNOR standard concerning the GMO detection [36]). Five different methods were used to get DNA with "potential" chemical differences, namely :

- CTAB method (CTAB)
- Phenol / chloroform method (PC)
- "High pure PCR template purification kit" (HP) method from Roche Applied Systems
- Association of the semi-automatic device "Kingfisher™ " and the "Genomic DNA purification Kit for Kingfisher" from ThermoLifeSciences (TLS).
- Association of the semi-automatic device "Kingfisher™ " from ThermoLifeSciences and the "Wizard® Magnetic DNA purification Kit for Food" from Promega (PFF)

The two first methods are based on fractionated precipitation and use of organic solvents. The other methods rely on the adsorption of DNA on beads coated with silicate.
For two of these extraction methods (CTAB and PFF), Rnase A digestion is performed for RNA degradation. In order to compare effect of RNA molecules in DNA extract, the PC and the HP extracts were also treated with RNase A (this digestion is usually not performed for these last procedures). PC and CTAB extraction protocols are those mentioned in the pre-normative CEN/TC 275/WG 11 N148 document [22] with minor modification (K_2EDTA is replaced by Na_2EDTA). The protocols for HP, PFF and TSL extraction are performed in accordance with the supplier's instructions [37, 38, 39].

A plasmid DNA purified on cesium chloride [40] was used as reference material. The pLec2 plasmid [41] which contains a specific insert from the soybean genome was then used as model to determine the amplificability of the soybean genomic DNA. The linearized pLec2 plasmid was used as reference for calibration. The initial concentration set for each DNA engaged in the PCR was set to 100,000 copies of the linear pLec2 plasmid.

General overview of the real time PCR

Real time PCR offers the possibility to monitor the production of amplicons during the whole reaction. Monitoring of the whole PCR allows drawing a characteristic curve that could be split in three main phases :

- A baseline where the exponential amplification is not visible
- An exponential amplification phase where the theorical amplification coefficient is a factor 2, meaning that at each cycle the copy number of an amplicon doubles.
- A linear and a plateau phases.

2.3.3.1.2 Results and discussion

A first observation resulting from our experiments (representing several runs and thousands of PCR reactions using two different real time PCR thermocycler : ABI 5700 and ABI 7000) relates to the general overview of the PCR kinetics. We did observe that the exponential phase (see general overview of real time PCR in materials and methods) could be split in two distinct phases. The first one, optional, has erratic features (for example an amplification coefficient larger than 2, which is probably an artefact linked to baseline setting). The second one is the real exponential phase. We therefore split the curves of each experiment in 4 phases :

Phase 1 : baseline (no visible exponential amplification)
Phase 2 : optional phase (visible or not) with erratic features for the exponential amplification
Phase 3 : real exponential phase
Phase 4 : linear and plateau phases

Comparison of different amplifications should be ideally performed during the phase 3. However, one should care on the fact that, as comparing numerous curves in phase 3, a common threshold should be set for all curves (for the same target). We thus introduced the

concept of "**consensus interval**" that defines the range of fluorescence values within which we can really compare the Ct for each curve.
The second part of these results relates to the PCR efficiency and the variability on quantitation. The PCR efficiency is expressed as an amplification rate : the theoretical amplification rate is 2, meaning that the copy numbers of an amplicon doubles at each cycle. The variability (all PCR were performed in 4 replicates) is expressed as a standard deviation SD on the Ct value (Ct = Cycle threshold is the number of cycles needed to reach a determined relative level of fluorescence during real time PCR). This SD is converted in Relative Standard Deviation -or coefficient of variation- (RSD in %) but associated to the copy number of the considered target amplified during PCR. The **table 19** shows these two parameters values observed for the different DNA extracts with pLec2 (linear or not) as reference DNA.

Table 19: Cf values calculated on each IRMM standard (CRM) and comparison samples (CS1 and CS2)

Run -->	1	2	3	4	Variability		
Calibrators -->	MTP1-DP1	MTP2-DP1	MTP1-DP2	MTP2-DP2			
Sample	Cf values for IRMM standards (CRM)				Mean Cf	SD Cf	RSD Cf (%)
CRM 0.1%	0.56	0.64	0.78	0.99	0.74	0.19	25.56
CRM 0.5%	0.57	0.61	0.71	0.59	0.62	0.06	10.08
CRM 1.0%	0.52	0.66	0.64	0.75	0.64	0.10	14.90
CRM 2.0%	0.61	0.57	0.63	0.70	0.63	0.06	9.05
CRM 5.0%	0.55	0.55	0.55	0.56	0.55	0.00	0.90
Sample	Cf values for Comparison Samples				Mean Cf	SD Cf	RSD Cf (%)
CS1 (1.3%)	0.56	0.58	0.62	0.65	0.60	0.04	6.69
CS2 (0.7%)	0.56	0.60	0.65	0.69	0.62	0.06	9.17

CRM : Certified Reference Material, Ref. IRMM410S.

Results in table 19 demonstrate that in the "non-consensus interval", the PC DNA extract treated with RNAse A has the highest (but absurd) amplification rate value even when compared to the linearized pLec2 plasmid. When suppressing the RNAse treatment, the same extract has the lowest amplification rate value. In the "consensus interval", the HP extract has the highest amplification rate value (close to the one gained outside the "consensus interval", indicating the robustness in the exponential phase for the HP extract). Concerning the variability, these results show that the RSDc (with c for copy number) is less than 25% (generally accepted as maximum variability value in GMO detection). Taking into account this parameter, all the extracts can then be accepted for quantitation. If we consider only the "consensus interval", the HP extract and the TLS extract have respectively the two best reproducibilities (lower RSDc). From the variability of the other extracts, it is difficult to rank them from the best to the worst one. In fact, some extracts have a low variability at the beginning of the "consensus interval" (Rn = 0.1) and a higher one at the end of this interval (Rn = 0.2) (e.g. PC with RNAse A extract). The contrary is also true (e.g. HP with RNAse A extract).
Altogether, the results obtained so far demonstrate the importance to work within an interval (called here "consensus interval") of the exponential amplification in real time PCR when comparing different samples. We also demonstrated that, for a defined concentration of the soybean DNA (100,000 copies of the soybean haploid genome), it possible to have similar efficiencies of amplification between genomic DNA and linear plasmid purified on cesium chloride. We could observe a positive effect of RNAse A digestion for the PC method. However, this effect has not been observed for the others methods (there is even a reverse effect on HP method).

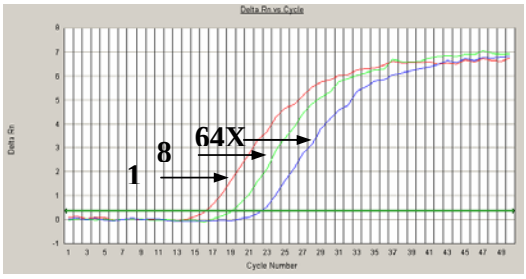
2.3.3.2 Influence of the inhibitory effect on GMO quantitation

One of the major problems in GMO quantitation is the variable quality of extracted DNA. Damaged DNA molecules or residual impurities can interfere with DNA amplification. The aim of these experiments is to analyse the amplification kinetics (efficiency and inhibitory effect) of different combinations of “matrix-DNA extraction methods”.

2.3.3.2.1 Materials and methods

Fourteen matrices were selected using as selection criterion the fact that PCR inhibition had been encountered in routine analysis with that sample or that kind of matrix. The selected matrices can be subdivided in four product types: **raw products** (maize leaves and kernels, fresh tomatoes), **processed products derived from soybean** (oilcake, proteins) or from **maize** (starch, gluten feed, silage, cornflakes) and four **compound feed** out of which two showed a strong inhibitory effect on PCR. Three DNA isolation methods were performed on each matrix: CTAB method (CTAB) [22], phenol-chloroform method (PC) [22], and Wizard® Magnetic DNA Purification System for Food method (from Promega) [38]. Three dilutions of each DNA extract were analysed by real time PCR with a universal plant target. In parallel, a plasmid DNA used as internal control was amplified through a specific target with or without the presence of the genomic extracts at different dilutions. Moreover absence of this target born by the plasmid had been checked for each DNA extract before this study. The **figure 27** shows two real time PCR patterns (one for the endogenous target, the other one for the external target) for a DNA extract with no inhibitory effect.

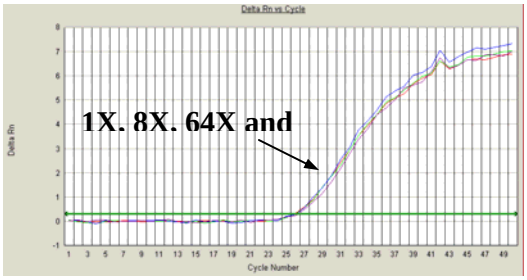
Figure 27: Examples of two real time PCR patterns for DNA extracts with no inhibitory effect



27a. Detection of an endogenous (universal plant) target on several dilutions of a genomic DNA extract (CTAB method) of maize leaves.

Real time PCR kinetics are parrallel but with a difference of 3 cycles (ΔCt = 3) between each DNA dilution (8-fold dilutions).

1x : 1x DNA extract
8x : 8x diluted DNA extract
64x : 64x diluted DNA extract



27b. Detection of an external (plasmid) target with addition of several dilutions of a genomic DNA extract (CTAB method) of fresh tomato.

Real time PCR kinetics for the plamid target are the same (the curves are superposed) with or without addition of genomic DNA in several dilutions from maize leaves.

pDNA : plasmid DNA without addition of DNA extract
1x : 1x DNA extract + pDNA
8x : 8x diluted DNA extract + pDNA
64x : 64x diluted DNA extract + pDNA

2.3.3.2.2 Results and discussion

The first observation of these results is that inhibition may be absent in samples with a high DNA content (e.g. genomic material extracted from maize kernels) while it may be present in samples with a low total DNA content (compound feed) especially if used undiluted. But the main observation in the context of real time PCR kinetics analysis is the existence of two principal types of inhibitory effects. The first one (type I) is characterized by decreased amplification efficiency during the exponential phase (generally together with some time-delay of the signal). While the second one (type II) consists simply in delayed signals but completely parallel to those without inhibition. These two types of inhibition kinetics are shown in **figures 28** and **29** together with at least a dilution devoid of any inhibitory effect.

Figure 28: Example of real time PCR patterns for DNA extracts with a ”type I” inhibitory effect

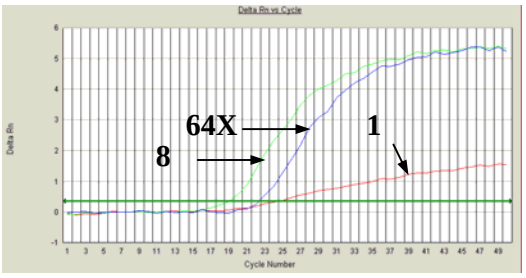


fig.28a: Detection of an endogenous (universal plant) target on several dilutions of a DNA extract (phenol-chloroform method) of soybean oilcake.

1x : 1x DNA extract
8x : 8x diluted DNA extract
64x : 64x diluted DNA extract

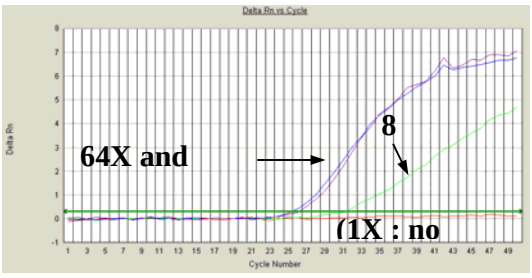


fig.28b: Detection of an external (plasmid) target with addition of several dilutions of a genomic DNA extract (phenol-chloroform method) of a compound feed.

pDNA : plasmid DNA without addition of DNA extract
1x : 1x DNA extract + pDNA
8x : 8x diluted DNA extract + pDNA
64x : 64x diluted DNA extract + pDNA

Figure 29: Example of real time PCR patterns for DNA extract with a ”type II” inhibitory effect

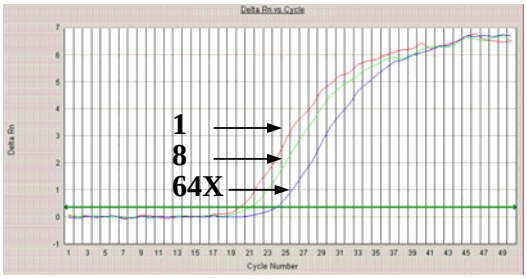


Fig. 29a: Detection of an endogenous (universal plant) target on several dilutions of a genomic DNA extract (phenol-chloroform method) of maize starch.

1x : 1x DNA extract
8x : 8x diluted DNA extract
64x : 64x diluted DNA extract

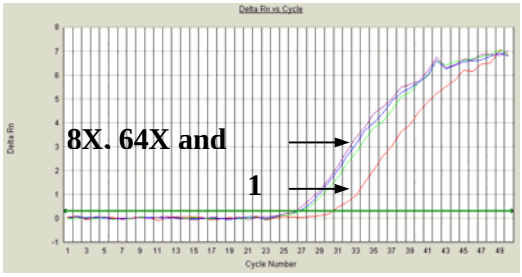


Fig.29b: Detection of an external (plasmid) target with addition of a genomic DNA extract (phenol-chloroform method)) of maize starch.

pDNA : plasmid DNA without addition of DNA extract
1x : 1x DNA extract + pDNA
8x : 8x diluted DNA extract + pDNA
64x : 64x diluted DNA extract + pDNA

Type II inhibition suggests that here the inhibitory effect probably only happens during the early cycles of the reaction when the exponential phase is not even visible in real-time PCR. While for type I, the inhibitory effect apparently goes on during the all reaction. That is why it can be hypothesized that the inhibition of the amplification in these two cases is due to molecules acting in different mechanisms. The main consequence of this statement is that when using single concentrations of extracts (in other words no dilution series) in real-time PCR, possible inhibition (especially type II) will be undetected and cause a bias for the GM quantitation purpose. The existence of two types of inhibition found during this task was communicated at a scientific meeting held in Prague [42].

The second part of these results focus on the different patterns of inhibitory effects observed for each DNA extract. **Table 20** synthesizes these different patterns.

Table 20: Overview of inhibitory effects observed on real time PCR performed on different DNA extracts obtained with three extraction methods.

Sample Name	Matrix	Phenol-chloroform extracts				CTAB extracts				Promega For Food extracts			
		Plant DNA target		External target		Plant DNA target		External target		Plant DNA target		External target	
		Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II
1	Maize kernels	0	Id	0/I(c/d)	Id	0	0/I (c/d)	I (c/d) (2)	0	0	0/I(c/d)	0	0/Ic
2	Maize leaves	0	0	Ic	Ic	0	0	0/Ic (2)	0	0	0	0	0/Ic
3	Fresh tomato	0	0	0/I(c/d)	0/I(c/d)	0	0/Ic	0	0	0	0/I(c/d)	0/Ic (2)	0
4	Soya oil cake	Ic	0/I (c/d)	Ic	Ic	0	0/Ic	I (c/d) (2)	0	0	0/Ic	0/Ic (2)	0
5	Soya proteins	Ic	I (c/d)	Ic	Ic	0	0/Ic	I (c/d) (2)	0	0	0/Ic	0	0
6	Maize starch	0	Ic	Ic	Ic	0	0/Id	I (c/d) (2)	0	0/Ic	Ic	Ic	Ic
7	Maize glutenfeed (I)	0/Ic (1)	I (c/d) (1)	I(c/d)	0/I(c/d)	0	0/Ic	I (c/d) (2)	0/Id	0	0/Id	0	0
8	Ensiled maize kernels	0	0	0/Id	0/Ic	0	0	I (c/d) (2)	0	0	0	0/Ic (2)	0
9	Maize glutenfeed (II)	0	Id	I (c/d) (1)	I (c/d) (1)	0	0	I (c/d) (2)	0	0	0	0	0/Ic
10	Corn flakes	0	Id	I (c/d)	I (c/d)	0	0	I (c/d) (2)	0	0	0	0	0/Ic
11	Compound feed (I)	Ic	Ic	Ic	Ic	0	0/Id	Ic (2)	0	0	0/Ic	0	0/Ic
12	Compound feed (II)	Ic	Ic	Ic	Ic	0		Ic (2)	0	0	0/Ic	0/Ic	0/Ic
13	Compound feed (III)	Ic	Ic	Ic	Ic	0/I(c/d)?	0/I(c/d)?	Ic (2)	0	0/I(c/d)	0/I(c/d)	0/Ic (2)	0
14	Compound feed (IV)	Ic	Ic	Ic	Ic	0	0/Id	0/Ic (2)	0	0	0/Ic	0/Ic (2)	0

Type I : "Type I" inhibitory effect.
Type II : "Type II" inhibitory effect.
I : Presence of an inhibitory effect of the considered type
0 : No inhibitory effect observed.
0/I : Slight inhibitory effect observed but may be not significant.
c : Inhibitory effect especially observed on concentrated extracts.
d : Inhibitory effect especially observed on diluted extracts or loss of targets by DNA sticking on used vials.
(c/d) : Inhibitory effect observed on concentrated and diluted extracts.
(1) Particular types of inhibition (see point “particular types of inhibition” for complementary results).
(2) Probably no significant "type I" inhibitory effect.

A first conclusion about the results of Table 20 is that the phenol-chloroform (PC) extraction protocol used gives raise to a much larger number of PCR inhibition than the CTAB protocol (CTAB) or the magnetic-beads protocol (PFF). The difference between PC and CTAB protocols may at least partially be explained by a more effective elimination of some macromolecules (essentially polysaccharides) during the CTAB extraction. Similarly, there is less inhibition problems with extracts of the magnetic beads system (PFF) compared to those obtained with the PC-protocol because of a larger elimination rate of proteins in the PFF protocol (use of a precipitation solution) and also because of the selectivity towards DNA of the used resin in the PFF system. The observed inhibition effect with PC extracts may

however be attenuated, if not eliminated, through a simple dilution of the extract but this is not valid for all the matrices (see specific inhibition case in the next paragraph).
A second observation is that with cases of clear inhibition (e.g. PC6, PC11, PC12, PC13) the inhibitory effect is generally more important on the concentrated extract. This may easily be explained by the higher concentration of inhibitors in such conditions. Sometimes however, inhibition seems to appear on the diluted extract. This effect on PCR may certainly in part be explained by an artefact linked to a loss of targets by sticking of DNA to the walls of the vial. Therefore, those cases where inhibition appears only or mainly with largely diluted extracts are to be considered with caution.
Finally two extracts (PC7 and PC9) prepared from maize gluten feed were studied in more detail due to their particular inhibition patterns. These special cases are described in the next paragraphs.

2.3.3.3 Particular inhibitory effects

The PC7 and PC9 extracts (both on maize gluten feed) were studied more extensively. Indeed these extracts showed type I and type II inhibition patterns but the type II inhibition seemed to increase with an increasing dilution of the extract. For these extracts type I inhibition was really clear when an exogenous target was amplified in the presence of the undiluted (1x) extract. Extract PC7 showed also a type I inhibition pattern when the endogenous target (universal plant target) was amplified.

2.3.3.3.1 Materials and methods

The experimental approach used here is identical to the one used in the previous experiments that made it possible to identify the inhibition patterns. Here however 22 serial dilutions (dilution factor of two between each dilution) were analysed (dilutions from 1 to 2^{21}). Like in the former experiments, inhibition was studied on an endogenous target (universal plant target) and on an exogenous target (amplification of a plasmid target added as external control). In this experiment, 7.5 µl of each dilution of DNA extract was studied by real-time PCR within a total volume of 25 µl. Dilutions were tried out in duplicate.

2.3.3.3.2 Results and discussion

Results of the performed experiments show that the serial dilutions may be divided into three great groups. A first group (dilutions 2^0 to 2^2) showed a type I inhibitory pattern. A second group (dilutions 2^3 to 2^{11}) showed a type II inhibitory pattern with a climax effect at the intermediate dilutions (dilutions 2^7 to 2^8). A third group (dilutions 2^{12} to 2^{21}) was free of any inhibition. **Figures 30** and **31** illustrate these effects. Figure 30 gives the respective Ct's reached during amplification of the endogenous target (universal plant target) in the presence of dilutions of extract PC7. Figure 30a gives the results for all the studied dilutions while figure 30b is limited to those Ct figures where no inhibition is observed. In these diagrams the inhibitory effect is characterized by a too large Ct in comparison to the expected Ct value.

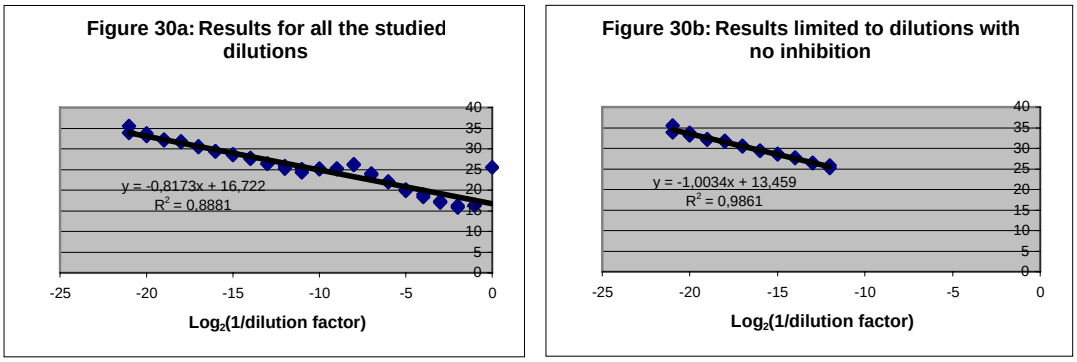


Figure 30: Ct values for the endogenous - universal plant - target on different dilutions of maize gluten feed (I) DNA obtained by phenol-chloroform extraction method (sample PC7).

As can be seen too in these figures, when the dilutions characterized by an inhibition are skipped (i.e. dilutions 2^0 to 2^{11}), the slope of the regression line (between the Ct and the logarithm of the concentration or logarithm of "1/Dilution factor") is affected and evolves from –0.8173 to –1.0034 (a figure close to the theoretical one, -1.0000 when using logarithms at base 2 and -3.3219 when using decimal logarithms). This last observation does not necessarily mean that the fitting is fine. This could also have been obtained through compensation effects. The quality of the fitting is expressed through the determination coefficient R^2 that should be as close as possible to one. Here the fitting is quite good as in Figure 30b, the R^2 parameter reaches 0.9826. Finally it should be stressed that these diagrams are only informative for type II inhibition (delayed Ct). For detection of a type I inhibition (reduced efficiency throughout the entire amplification) it is required to analyse the kinetics of each dilution of the extracts (data not considered in this report).

Figures 31a and 31b illustrate the inhibitory effects observed on an exogenous target (Mon810 target in pMon810 plasmid) added to several dilutions of the DNA extract.
Figure 31a represents the Ct figures obtained during amplification of the exogenous target added to several dilutions of the DNA extract. In this diagrams type II inhibitory effect is characterized by Ct values exceeding the one obtained when the plasmid is amplified without the presence of the extract (Ct of ~15.6). Figure 31b shows the different types of inhibition per dilution group.

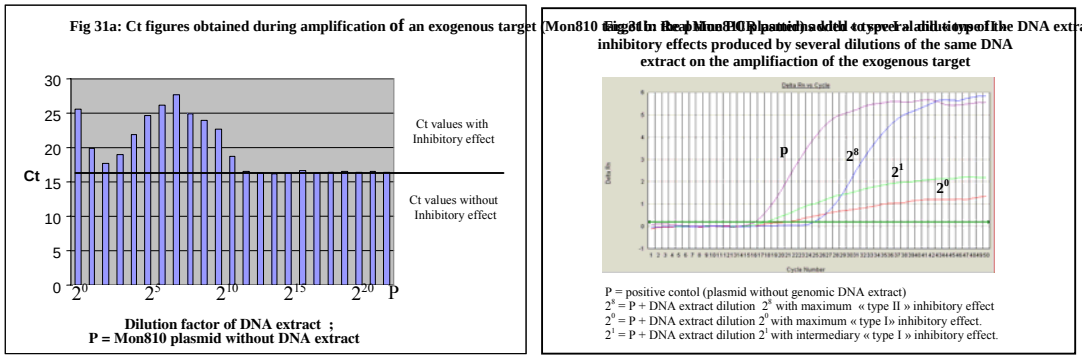


Figure 31: Inhibitory effects observed on an exogenous target (Mon810 target in pMon810 plasmid) added to several dilutions of maize gluten feed (I) DNA obtained by phenol-chloroform extraction method (sample PC7).

These experiments on different targets (an endogenous and an exogenous one) show that similar inhibitory patterns of type I and type II are observed at the same dilutions of the extracts whatever the kind of target used. From these results we infer the hypothesis that some molecules acting through two different mechanisms are present in the extract.

2.3.3.4 Capacity of DNA extracts to be amplified by real-time PCR: determination of the detection and quantitation ranges of soybean specific target in several extracts of certified reference flour

2.3.3.4.1 Objective

The aim of this part is to define the detection and quantitation range of a specific target (lectin) obtained through several DNA extraction methods from a given model matrix (certified reference flour). Determination of the upper limit range concerning the amount of DNA that can be used in PCR is essential as it is directly linked to the lowest values of relative limit of detection (rLOD) and limit of quantitation (rLOQ) and therefore to the performance of the extraction method itself. Therefore the PCR-usable quantity of DNA obtained from a matrix is a fundamental quality criterion of a considered DNA extraction method.

2.3.3.4.2 Methodology

Model matrix and DNA extraction

The model matrix used here is the soybean reference flour (1% Roundup Ready soybean, ref. IRMM 410S-3). Three extraction methods were considered for the analysis: PC, CTAB and PFF. Two test portions of 200 mg were used for each extraction. After extraction and purification the DNA yielded by the CTAB and PC methods were suspended in 200 µl of TE. The DNA obtained through the PFF method was eluted with 200 µl of PCR-grade water. DNA concentrations of these several extracts were determined by quantitation of the soybean lectin target in real-time PCR by using a CTAB extract as calibrant after determination of its own concentration by spectrophotometry.

Real time PCR experiments

A first experiment consisted of serial dilutions of the extracts of each test portion (tenfold dilutions with a dilution factor from 10^0 to 10^6) of which 10 µl were submitted to amplification of the lectin target. PCR was performed in triplicates on each test portion. The aim of this experiment is to assess the detection and quantitation range of the lectin target in the several considered extracts.
Acceptance criteria for a given dilution of an extract in terms of qualitative detection is the presence of an amplification signal in each of the triplicates related to that dilution. While for the quantitative range, 2 parameters are taken into consideration: possible presence of inhibition and variability of the quantitative response. The amplification efficiency was measured on a per curve basis within the “consensus interval” (see definition of the “consensus interval” at § 2.3.3.1.2 page 56) in order to distinguish a possible type I inhibition.

While presence of a possible type II inhibition was checked by looking at the Ct intervals between the successive dilutions (see § 2.3.3.2.2 page 59 for definition of type I and type II inhibition). A dilution point of an extract was considered to be still within the quantitative range if no inhibition was observed and if variability on the quantitative response did not exceed 30% in RSD of the copy number (several international standards and recommendations accept a RSD of 25 to 33% [e.g. 43]).
With the above detailed selection criteria it was possible to define per extraction type which were the detection and quantitation range. Once this was done, the amplification efficiency (A_r) was also established by means of the regression curve (between Ct and logarithm of the copy number) limited to those dilution points considered as acceptable from a quantitative point of view. The so obtained figure was then compared to the mean amplification efficiency (A_c, i.e. the mean of the efficiencies measured on a per curve basis for each dilution point of an extract).
A second experiment aimed at defining more precisely the maximal amount of DNA that could still be used in real time PCR for the several considered extraction methods. Therefore 6 serial dilutions of these extracts were performed (twofold dilutions with a dilution factor going from 2^0 to 2^5) and analysed in PCR. Acceptance criteria for qualitative and quantitative PCR were the same as in the previous experiment. With these figures the rLOD and rLOQ (with "r" for relative) were determined considering an absolute LOD of 20 copies (as recommended by the AFNOR standard XP V 03-020-1 [36]) for the transgenic target and an absolute LOQ between 20 and 100 copies (in fact the AFNOR standard states "*quelques dizaines de copies*") for the transgenic target.

2.3.3.4.3 Results and discussion

Amplification efficiency and variability of the quantitative response

Table 21 summarizes the several data linked to amplification efficiency, variability of the quantitative response and presence of inhibition during the assessment of the PCR results of the several dilution points per extraction method (this table considers only the first experiment with tenfold dilutions). The most interesting observation to be stressed here handles about amplification efficiency. One can see from the obtained results that the mean efficiency per dilution (A_c) is always lower than the efficiency (A_r) that can be derived from the standard curve. This is probably reflecting the fact that the latter figure is more global. This means that analysis of the efficiency on a curve by curve basis is important to detect a type I inhibition but the efficiency derived from the slope of the calibration curve gives a more general appraisal of the amplification capacity of an extract
Concerning the variability of the quantitative response, one can see that it shows some trend to increase with the importance of the dilution factor of the extracts obtained with the CTAB or PFF method. While for the PC method there is no such trend and variability appears to be much more random. This last observation might be linked to some possible PCR interfering molecules present in larger amount in the PC extract when used at low dilution rates and thus resulting in less reproducible quantitations.

Tableau 21:. Data linked to amplification efficiency, variability of the quantitative response and presence of inhibition during the assessment of the PCR results of the several dilution points per extraction method (this table considers only data from the first experiment with tenfold dilutions).

DNA extract	DNA Quantity (pg)	Copy number of haploid genomes	Curve by curve analysis				Amplification rate analysis by			
			RSD Q(%)	A_c	Mean A_c	Mean A_c (%)	Slope	R^2_{slope}	A_r	A_r (%)
CTAB1	800000	696000.0	Type II inhibitory effect : quantitation not possible							
	80000	69600.0	**6.44**	**1.80**	**1.81**	**90.26%**	**-3.5127**	**0.9981**	**1.93**	**96.31%**
	8000	6960.0	**4.47**	**1.77**						
	800	696.0	**7.40**	**1.78**						
	80	69.6	**19.95**	**1.86**						
	8	7.0	DNA detected but under limit of quantitation							
	0.8	0.7	DNA concentration under limit of detection							
CTAB2	800000	696000.0	Type II inhibitory effect : quantitation not possible							
	80000	69600.0	**6.95**	**1.73**	**1.78**	**89.12%**	**-3.4347**	**0.9989**	**1.95**	**97.75%**
	8000	6960.0	**4.01**	**1.78**						
	800	696.0	**13.78**	**1.84**						
	80	69.6	**8.55**	**1.78**						
	8	7.0	DNA detected but under limit of quantitation							
	0.8	0.7	DNA concentration under limit of detection							
PC1	800000	696000.0	Type I inhibitory effect : DNA not amplified							
	80000	69600.0	**16.66**	**1.76**	**1.81**	**90.71%**	**-3.4313**	**0.9963**	**1.96**	**97.81%**
	8000	6960.0	**6.70**	**1.85**						
	800	696.0	**4.73**	**1.88**						
	80	69.6	**19.22**	**1.77**						
	8	7.0	DNA detected but under limit of quantitation							
	0.8	0.7	DNA detected but under limit of quantitation							
PC2	800000	696000.0	Type I inhibitory effect : DNA not amplified							
	80000	69600.0	**28.40**	**1.78**	**1.76**	**88.08%**	**-3.3753**	**0.9961**	**1.98**	**98.91%**
	8000	6960.0	**11.24**	**1.77**						
	800	696.0	**2.46**	**1.77**						
	80	69.6	**4.01**	**1.72**						
	8	7.0	DNA detected but under limit of quantitation							
	0.8	0.7	DNA concentration under limit of detection							
PFF1	160000	139200.0	Type I inhibitory effect : DNA not amplified							
	16000	13920.0	**4.14**	**1.86**	**1.86**	**93.00%**	**-3.604**	**0.998**	**1.89**	**94.72%**
	1600	1392.0	**6.70**	**1.79**						
	160	139.2	**20.99**	**1.92**						
	16	13.9	**16.49**	**1.87**						
	1.6	1.4	DNA concentration under limit of detection							
	0.16	0.1	DNA concentration under limit of detection							
PFF2	160000	139200.0	Type I inhibitory effect : DNA not amplified							
	16000	13920.0	**4.80**	**1.83**	**1.81**	**90.26%**	**-3.6**	**0.9976**	**1.90**	**94.79%**
	1600	1392.0	**3.20**	**1.66**						
	160	139.2	**20.22**	**1.91**						
	16	13.9	**19.61**	**1.83**						
	1.6	1.4	DNA concentration under limit of detection							
	0.16	0.1	DNA concentration under limit of detection							

RSD Q (%) : Relative Standard Deviation on DNA quantity.
A_c : Mean of the amplification rates (n=3) calculated curve by curve during the exponential "consensus" interval.
Mean A_c : Mean A_c calculated from data of DNA concentrations considered as acceptable for quantitation (i.e. A_c values on grey background).
Mean A_c (%) : PCR efficiency corresponding to Mean A_c.
Slope : slope of the calibration curve calculated (in the "consensus interval") from DNA concentrations considered as acceptable for quantitation.
R^2_{slope} : Coefficient of determination of the slope.
A_r : Amplification rate calculated from the slope.
A_r (%) : PCR efficiency corresponding to A_r.

Determination of detection and quantition ranges

Table 22 gives the considered range of DNA amounts (expressed as the quantity of DNA involved in a 25 µl PCR mix) and - per extraction method - the detection and quantitation range with the corresponding relative detection and quantification limits. These figures are based on the results of both experiments, the one with the tenfold dilutions and the one with the twofold dilutions focusing on the upper limit of the range.
The considered amounts of DNA go from 800 ag to 800 ng (representing respectively from 0.7 to 696 000 haploid genomes of soybean) for the CTAB and PC extracts. For PFF these figures go from 160 ag to 160 ng for the DNA amount (i.e. respectively 0.14 to 139 200 haploid genomes of soybean) because the magnetic beads method has a lower DNA yield (at least when working in similar conditions i.e. same size of test portion and same final volume in which DNA is recovered).

A first important conclusion is that the relative limits of detection and quantification are far below the 0.9% threshold as considered on a per ingredient basis for food and feed. Nevertheless, the $rLOQ_{100}$ of 0.14% for PFF extracts is already somewhat high if 0.3% or even 0.5% thresholds should have to be considered like once discussed for seeds.
The obtained results clearly show that the CTAB method is the most favourable one in terms of PCR-usable amounts of DNA (and therefore the lowest rLOD and rLOQ are linked to it).

Table 22: Detection and quantitation range of the several considered DNA extraction methods on certified reference soybean flour as matrix (the DNA amounts given are those involved in 25 µl PCR mix).

DNA Extraction method	Global[(1)] analysed range	Global[(1)] detection range	Global[(1)] quantitation range	$rLOD_{20}$ (%GMO)[(2)]	$rLOQ_{20}$ (%GMO)[(3)]	$rLOQ_{100}$ (%GMO)[(4)]
CTAB	0.8pg to 800ng	8pg to 800ng	80pg to 400ng	0.003%	0.006%	0.029%
	0.7c to 696000c	7c to 696000c	70c to 348000c			
PC	0.8pg to 800ng	8pg to 400ng	80pg to 200ng	0.006%	0.011%	0.057%
	0.7c to 696000c	7c to 348000c	70c to 174000c			
PFF	0.16pg to 160 ng	16pg to 80ng	16pg to 80ng	0.029%	0.029%	0.144%
	0.14c to 139200	14c to 69600c	14c to 69600c			

[(1)] This table considers the data of the two experiments (tenfold dilutions experiment and twofold dilutions experiment).
c :Copies expressing the number of equivalent haploid genomes present in the considered quantity of DNA.
[(2)] $rLOD_{20}$ (%GMO) : relative limit of detection with an absolute limit of detection for GM marker = 20 copies.
[(3)] $rLOQ_{20}$ (%GMO) : relative limit of quantitation with an absolute limit of quantitation for GM marker = 20 copies.
[(4)] $rLOQ_{100}$ (%GMO) : relative limit of quantitation with an absolute limit of quantitation for GM marker = 100 copies.

2.3.3.5 Study on the stability of the extracted DNA

2.3.3.5.1 Objective

A good conservation of the extracted DNA is one of the problems that may be encountered during detection and quantitation analyses of GMOs. This concern exists as well for DNA standards used as positive controls (qualitative analyses) as with calibrators (quantitative analyses) but also with the DNA extract of the sample to be analysed. The ISO/CEN and AFNOR standards are stressing the fact that the extracted material should be stable without being freezed during the whole analysis. Practically however, freezing of samples is frequently performed in the laboratories especially if the analysis extends over several days (which may happen when the decision tree used is very progressive, first screening then identification and finally quantitation). Risks of DNA degradation are linked to freezing-thawing cycles. Two major consequences may be expected: i) the process will affect the number of copies of the calibrants; ii) the process will reduce the performance of the methods as it results in an increase of LOD and LOQ. The objective of this part is to measure more precisely the impact (in absolute value, as in relative value it may partly be compensated) of repetitive freezing-thawing cycles on DNA extracts stored at different concentrations.

2.3.3.5.2 Methodology

The chosen matrix is – once again – soybean flour (mean particle size below 500 µm) from which two mother solutions of DNA were prepared, one suspended in water the other one in TE buffer. The used DNA extraction method was the CTAB-method (see previous §). After determination of the DNA content by spectrophotometry, the mother solutions were diluted (in water or in TE) in a number of working solutions containing 0.1 ng, 1 ng, 10 ng or 100 ng of DNA per µl (corresponding respectively in number of haploid soybean genomes going from 87 to 87 000 when using the conversion data available in the AFNOR standard [44]). Each working solution was subdivided in eight aliquots in order to submit them to an increasing number of freezing-thawing cycles (i.e. for aliquot 1: one freezing, aliquot 2: two freezings, … and aliquot 8: eight freezings). In what follows the numbers of the aliquots will thus also represent the number of freezing-thawing cycles to which the aliquot was exposed. The several aliquots submitted to their freezing cycles were then analysed by real-time PCR in order to measure the impact of the repetitive freezing cycles on the amount of targets that can still be used in PCR. The PCR was performed in triplicate on each aliquot. In the analysis of the results the amount of PCR-usable targets in the aliquots of a same working solution (corresponding to a defined DNA concentration) were compared by using aliquot nr 1 as reference and setting its number of targets as 100%.

2.3.3.5.3 Results and discussion

Table 23 gives an overview of the obtained results in this stability experiment. Part A is devoted to DNA extracts suspended in water and part B to extracts suspended in TE buffer. Each part is itself divided in raw data and corrected data. The raw data report the measured Ct's and the derived copy numbers expressed here in % of the copy number of aliquot 1 for each working solution. While for the corrected data the Ct's and copy numbers in % are those

deriving from a trend curve established, per working solution, by regression between the Ct’s and the number of freezing cycles. The slope of the regression curve and the determination coefficient R^2 are also given in the aforementioned table. Moreover from this trend curve a mean loss of material per freezing cycle could be calculated and is also given per working solution.

Table 23: Overview of the remaining DNA amounts measured by real time PCR after several (from 1 to 8) freezing/thawing cycle(s) in PCR-grade water (a) and in TE buffer (b).

a

H_2O	Raw data							
#	100ng DNA		10ng DNA		1ng DNA		0.1ng DNA	
	Mean Ct (n=3)	% of remaining DNA(1)	Mean Ct (n=3)	% of remaining DNA(1)	Mean Ct (n=3)	% of remaining DNA(1)	Mean Ct (n=3)	% of remaining DNA(1)
1	22.29	100.00%	26.35	100.00%	30.24	100.00%	33.89	100.00%
2	22.32	97.72%	26.24	108.17%	29.94	123.11%	33.63	120.30%
3	22.38	94.17%	26.79	73.88%	31.38	45.48%	35.21	40.15%
4	22.30	99.08%	26.59	84.87%	30.84	65.98%	34.94	48.41%
5	22.55	83.70%	26.83	71.70%	31.07	56.12%	35.27	38.60%
6	22.42	91.38%	26.81	72.53%	31.91	31.35%	36.10	21.71%
7	22.43	90.75%	26.71	77.74%	31.17	52.36%	35.23	39.59%
8	22.43	90.96%	26.81	72.87%	31.26	49.43%	34.01	92.23%
	Correted data							
#	100ng DNA		10ng DNA		1ng DNA		0.1ng DNA	
	Slope	0.0222	Slope	0.0703	Slope	0.1801	Slope	0.1407
	R^2 Slope	0.4091	R^2 Slope	0.56	R^2 Slope	0.4865	R^2 Slope	0.1633
	Ct	% of remaining DNA(1)	Ct	% of remaining DNA(1)	Ct	% of remaining DNA(1)	Ct	% of remaining DNA(1)
1	22.31	100.00%	26.39	100.00%	30.35	100.00%	34.29	100.00%
2	22.33	98.47%	26.46	95.24%	30.53	88.26%	34.43	90.71%
3	22.36	96.97%	26.53	90.71%	30.71	77.91%	34.57	82.28%
4	22.38	95.49%	26.61	86.40%	30.89	68.76%	34.71	74.63%
5	22.40	94.03%	26.68	82.29%	31.07	60.69%	34.85	67.70%
6	22.42	92.59%	26.75	78.38%	31.25	53.57%	35.00	61.41%
7	22.45	91.18%	26.82	74.65%	31.43	47.28%	35.14	55.70%
8	22.47	89.79%	26.89	71.10%	31.61	41.73%	35.28	50.53%
	Lost DNA(1)	1.53%	Lost DNA(1)	4.76%	Lost DNA(1)	11.74%	Lost DNA(1)	9.29%

b

TE	Raw data							
#	100ng DNA		10ng DNA		1ng DNA		0.1ng DNA	
	Mean Ct (n=3)	% of remaining DNA(1)	Mean Ct (n=3)	% of remaining DNA(1)	Mean Ct (n=3)	% of remaining DNA(1)	Mean Ct (n=3)	% of remaining DNA(1)
1	21.94	100.00%	25.45	100.00%	28.97	100.00%	32.01	100.00%
2	21.93	100.46%	25.48	97.94%	28.95	101.40%	32.11	93.74%
3	21.87	104.97%	25.49	97.72%	29.02	96.82%	32.26	84.28%
4	21.98	97.27%	25.51	96.15%	29.07	93.30%	32.48	72.53%
5	22.03	93.74%	25.49	97.27%	29.15	88.27%	32.17	89.50%
6	22.10	89.30%	25.66	86.85%	29.48	70.22%	32.71	61.84%
7	22.07	91.38%	25.56	93.09%	29.43	72.53%	32.44	74.40%
8	22.01	95.04%	25.71	83.70%	29.61	64.32%	33.29	41.37%
	Corrected data							
#	100ng DNA		10ng DNA		1ng DNA		0.1ng DNA	
	Slope	0.0232	Slope	0.0316	Slope	0.0993	Slope	0.1383
	R^2 Slope	0.5414	R^2 Slope	0.7082	R^2 Slope	0.8894	R^2 Slope	0.6775
	Ct	% of remaining DNA(1)	Ct	% of remaining DNA(1)	Ct	% of remaining DNA(1)	Ct	% of remaining DNA(1)
1	21.91	100.00%	25.43	100.00%	28.86	100.00%	31.95	100.00%
2	21.93	98.40%	25.46	97.83%	28.96	93.35%	32.09	90.86%
3	21.95	96.83%	25.50	95.71%	29.06	87.14%	32.23	82.55%
4	21.98	95.29%	25.53	93.64%	29.16	81.34%	32.36	75.01%
5	22.00	93.77%	25.56	91.61%	29.26	75.93%	32.50	68.15%
6	22.02	92.27%	25.59	89.63%	29.36	70.88%	32.64	61.92%
7	22.05	90.80%	25.62	87.68%	29.46	66.17%	32.78	56.26%
8	22.07	89.35%	25.65	85.79%	29.56	61.77%	32.92	51.12%
	Lost DNA(1)	1.60%	Lost DNA(1)	2.17%	Lost DNA(1)	6.65%	Lost DNA(1)	9.14%

Number of freezing/thawing cycles
(1) Lost DNA per freezing/thawing cycle

The obtained results clearly show a general trend of decrease of the amount of PCR-usable targets with an increasing number of successive freezings. This trend is more pronounced when the concentration of DNA is lower (there is only one exception observed with the 1 ng/µl working solution in water in which degradation rate is more pronounced then for the working solution at 0.1 ng/µl). Nevertheless it should be stressed that the trend curves used show sometimes low determination coefficients (R^2 from 0.1633 at 0.1 ng/µl DNA in water to 0.8894 for 1 ng/µl DNA in TE). This means that the trend curve does not always completely explain the observed decrease. Nevertheless it is clear that a general trend of loss of DNA targets is observed when freezing-thawing cycles are repeated and this loss is higher when the DNA stock concentration is lower.
It should be noticed that quantifying an extract for its GMO content is done in a relative way using two targets, therefore maybe their absolute losses are comparable and probably partially compensated when expressed in a relative way so that the final quantitative results are not affected in the same way. Nevertheless the LOD and LOQ of the quantitation method are directly linked to the absolute amounts of targets.

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3 CONCLUSIONS

The current OSTC project addresses normative solutions to several problems associated with detection, authentication and quantification of GM plants and derived products used in the food/feed processing area.

One first goal of the project is the concept of using GMO markers cloned into plasmids as qualitative and /or quantitative reference material (theme 1).

We also address the issue of the increasing GMOs to come on the European market. To face this problem, we proposed to develop and validate a tool allowing the simultaneous detection of different GMOs in a single sample: the qualitative GMO chips (theme 2)

Finally, the quality of DNA engaged in the developed methodologies is critical to the success of DNA detection based protocols. We thus proposed to described if possible some « chemical descriptors » that could correlate with high PCR efficiency (theme 3).

Theme 1: Plasmid as GMOs reference material (partners #1, #2 and #5)

Validation of analytical methods does require reference material for each GMO to trace and quantify. There is a general lack of such material in due time. Among these reasons, it is extremely difficult for the companies to produce enough reference material of well characterized composition, and it takes a long time for Official producers such as the IRMM of Geel to set up and deliver Certified Reference Material necessary for accredited laboratories. To cope with the urgent needs of competent authorities in qualitative reference material for screening purposes and gain access to a cheap and stable delivery thereof, we proposed to refer qualitative screenings to genetic markers cloned into plasmids, the plasmids being the fastest, cheapest and easiest way to deliver genetic markers of 0.1 to 12Kbases length. It was also proposed by ISSP and CLO to build up a bank of such genetic markers.

The output of the work achieved in the frame of the project can be translated in a number of milestones obtained:

- Development of 151 plasmid containing different DNA markers allowing the identification of GM plant and/or derived products in feed/foodstuff. These DNA markers are event or construct specific. We also include all screening markers for the detection of GMOs authorized in the EU, namely 35S promoter, Nos and 35S terminator (task A1 under Theme 1).
- Establishment of a new, European ENGL database of plasmid markers. 22 pENGL are deposit in the existing BCCM^{TM}/LMBP database as private deposit (Task A2 under theme 1). The certificate of the deposited plasmid is provided in the annexe of the present report. Bacterial culture, plasmid DNA marker, primer pair, TaqMan probe as well as a complete scientific dossier, for each record in the database are available.
- Availability of three endogenous PCR assays, and ten event-specific PCR assays for nine commercial events. Each assay consists of a specific primer pair, a fluorescent Taqman probe, and optimized conditions of real-time PCR amplification.
- Evidence on the commutability of plasmid DNA calibrators with genomic DNA standards, proven in an extensive comparative study between three different types of calibrators.
- Insight in conversion factors between genome/genome percentages and seed/seed percentages, usable for quantification of GMO contents in seed lots. ‘Lower real-time

working thresholds’ available for quantification of T25 in seeds by means of real-time PCR, and allowing to compare with seed/seed thresholds established in legislation.

- Validation under ISO17025 norms of the use of a 5 screening pENGL **(pENGL-02-019, pENGL-03-010, pENGL-02-014, pENGL-02-015, pENGL-02-002)** using a SYBRgreen screening plate for qualitative purposes. Further, these same plasmids were mix. This qualitative pENGL screening mix was tested at a European level (task A4 under theme 1). The final report for this feasibility study will be available for the next plenary meeting of ENGL next April 2005.
- Results of the different advances of this work have been published and/or reported during workshop or international congress. The list is provided hereafter.

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Theme 2: Qualitative biochip for GMO detection (partners #1 and #3)

To face the increase of GMOs, which will enter the EU, we develop a tool allowing in a one shot experiment the detection and identification of numerous GMOs present in a single sample. The so called « qualitative GMO biochip » is planed to replace the routine qualitative PCR for detection and/or identification of GMO in food/feedstuff. According to the task B1 described under theme 2, partner #3 (FUNDP) complete the existing biochips (previous OSTC report) with plant species capture probe. This new chips was produced and partially validated by partner #3 (task B3 under theme 2). Full validation was expected to be performed by ISP (partner #1). However, due among other to the cost of the technique, further improvement of this last chips appears to be necessary. All in all, the qualitative biochip is not

yet operational. The possible validation expected by the JRC-Ispra (taskB4 under theme 2) could not occur.
However, at the time of the redaction of the present report, the full validation of the chips is ongoing since the different problems seem to be solved.

Theme 3: Analytical control of parameters influencing GMOs quantitation (partners #4 and #5) : Characterization of DNA extracts

One of the major problems regarding detection of transgenic DNA (or other targets) is the “variable quality” of extracted DNA. Damaged DNA, residual impurities or insufficient yields will totally or partially prevent DNA amplification, preventing and impairing real-time PCR. Moreover, lack of purity can lead to high variability.
We tried to estimate different methods to improve the efficiency of PCR (task C2 under theme 3). Globally, from the work carried out during the entire project, it is not obvious to define method to define “chemical descriptors” for DNA quality, mainly because the nature of genomic DNA extracts is far too heterogeneous. However, we clearly show that PCR failure can be explained by the presence of matrix-dependent contaminants that could be isolated by the use of anion-exchange cartridges. Among the different extraction method used, it appears that the phenol/chloroform procedure is not advisable for extraction of maize kernels, leaves or compound feed but it might be interesting to extract DNA from fresh tomatoes and gluten feed I. CTAB extraction procedure is advisable for most matrices except for fresh tomatoes and soybean proteins. Actually, CTAB is particularly efficient to separate DNA from acidic polysaccharides particularly abundant in plants. We also showed that the addition of an RNase digest-step in the phenol-chloroform extraction method should be a standard procedure and that purity of extracts is greatly improved when this step is carried at the end of the procedure
Due to lack of time, the project of a collaborative validation study for the extraction of DNA could not be achieve.

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