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Development of a Molecular Platform for GMO Detection in Food and Feed on the Basis of "Combinatory qPCR" Technology

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1. Introduction

Fifteen years after the first commercialisation of biotech crops, the global area of their cultivation comprises more than one billion hectares. The increase in the area between 1996 and 2010 is 87-fold which makes biotech crops the fastest adopted technology in modern agriculture (James, 2010).

In 2010, 184 Genetically Modified (GM – see glossary) events, representing 24 crops have already received worldwide regulatory approval. To date, 29 countries have cultivated GM crops, whereas 59 countries have granted regulatory approvals for their import for food and feed use and release into the environment. The six main countries cultivating GM crops are USA, Brazil, Argentina, India, Canada and China. In the EU the cultivation area of biotech crops amounts only 0,1% of the cultivation area reaching 125 million hectares in 25 countries (Stein & Rodriguez-Cerezo, 2009). The most important biotech crop is soybean (50% of the biotech crops cultivation area), followed by maize (31%), cotton (14%) and oilseed rape (4%) (James, 2010).

Herbicide tolerance and insect resistance are the main traits used in the first generation of GM crops. After 2009, many GM events conferring novel traits have entered the regulatory system. New traits were introduced in soybean, maize, cotton and oilseed rape. The second generation of traits comprises altered crop composition, new herbicide tolerances, virus and nematode resistance and abiotic stress tolerance. Furthermore, new crops such as potato and rice were approved in different countries (Stein & Rodriguez-Cerezo, 2009). Moreover, gene stacking is a trend that is likely to increase in the near future. There are new events containing up to four stacked traits in the regulatory pipeline. A maize stacked event containing up to eight traits is in an advanced research and development stage (Dow AgroSciences SmartStax® platform; James, 2010).

In the EU until 2010, 39 events were authorised for import and processing in food and feed and two for cultivation. This includes 23 maize events from which 12 containing double and triple stacked traits, seven cotton events from which two containing stacked traits, four oilseed rape events, three soybean events, one potato and one sugar beet event. A detailed list of the EU-authorised GM events per crop with their main traits is presented in table 1.

Another tendency is that new GM events are not solely developed and commercialised by international biotech companies anymore, but also by scientific governmental institutions. Many of these GM events are commercialised by Asian national research centres (e.g. China, India) and are intended for the local markets. However, as many food and feed materials are imported in the EU from third party countries, events that are not submitted for authorisation in the EU (unauthorised GMO or UGM) might accidentally end up into in the food and feed chain (Stein & Rodriguez-Cerezo, 2009).

In reaction to the public concern about the presence of Genetically Modified Organisms (GMO – see glossary) in the food chain, many countries have adopted a specific legislation with respect to the introduction of GMO on their market. The legislation requirements vary from country to country, but there are some common elements such as case by case safety assessment, distinction between contained use and release into the environment and a distinction between cultivation and use as raw products in processing. Commonly recognised is the concept of substantial equivalence (Shauzu, 2001). In many regulatory systems tolerances or labelling thresholds, varying between 0.9 and 5%, were introduced.

The EU legislation on GMO is complex and consists of several core elements: a pre-authorisation safety assessment, use of a labelling threshold, strict requirements for traceability of the GM products along the food chain and post-market monitoring. Labelling and traceability of new GM products are regulated mainly under Commission Regulations 1829/2003 and 1830/2003. For all events submitted under EC/1829/2003 a safety assessment is performed by the European Food Safety Authority (EFSA- see glossary). Food, feed and environmental risks are evaluated based on the data provided by the company requesting authorisation of a GM product. The food and feed safety assessment includes several issues such as allergenicity, toxicology, nutritional characteristics and post-market monitoring of the GM food and feed. The environmental risk assessment includes evaluation of the potential of gene transfer, interaction of the GM plant with target and non-target organisms and monitoring (EFSA, 2011).

A very important issue is the molecular characterisation of the GM event. The objective of this characterisation is to obtain information on the introduced trait or genetic modification and to assess if unintended effects due to the genetic modification have taken place (Organisation for Economic Co-operation and Development [OECD], 2010). The molecular characterisation is an evaluation of relevant scientific data on the transformation process and vector constructs used, inserted transgenic sequences, copy number of the inserts, presence of partial copies, expression of the transgenic protein, stability and the inheritance of the transgenic insert (EFSA, 2011). The information on the elements introduced in the GMO as well as the sequence information on the junction regions between the plant genome and the transgenic insert are an essential part as they are related to the development of detection methods.

Transformation event (Unique identifier)	Trait	Transformation event (Unique identifier)	Trait
Maize single events			
Bt11 **(SYN-BT Ø11-1)**	Insect resistance Herbicide tolerance (glufosinate)	**DAS59122** **(DAS-59122-7)**	Insect resistance (Coleopteran insects) Herbicide tolerance (glufosinate)
DAS1507 **(DAS-Ø15Ø7-1)**	Insect resistance (Lepidopteran insects) Herbicide tolerance (glufosinate)	**GA21** **(MON-ØØØ21-9)**	Herbicide tolerance (glyfosate)
MON810 **(MON-ØØ81Ø-6)**	Insect resistance (Lepidopteran insects)	**MON863** **(MON-ØØ863-5)**	Insect resistance (Coleopteran insects)
T25 **(ACS-ZMØØ3-2)**	Herbicide tolerance (glufosinate)	**NK603** **(MON-ØØ6Ø3-6)**	Herbicide tolerance (glyfosate)
MON88017 **(MON-88Ø17-3)**	Insect resistance (Coleopteran insects) Herbicide tolerance (glyfosate)	**MIR604** **(SYN-IR6Ø4-5)**	Insect resistance (Coleopteran insects)
MON89034 **(MON-89Ø34-3)**	Insect resistance (Lepidopteran insects)	*Bt176* *(SYN-EV176-9)*	*Insect resistance (European corn borer) Herbicide tolerance (glufosinate)*
3272 maize *(SYN-E3272-5)*	*Altered composition (increased a-amilase content)*	*MIR162* *(SYN-IR162-4)*	*Insect resistance (Lepidopteran insects)*
98140 *(DP-098140-6)*	*Herbicide tolerance (ALS-inhibiting herbicides)*		
Maize stacked events			
DAS1507xNK603 **(DAS-Ø15Ø7-1xMON-ØØ6Ø3-6)**	Insect resistance (Coleopteran insects) Double herbicide tolerance (glufosinate and glyfosate)	**NK603xMON810** **(MON-ØØ6Ø3-6 x MON-ØØ81Ø-6)**	Insect resistance (Lepidopteran insects) Herbicide tolerance (glyfosate)
DAS59122xNK603 **(DAS-59122-7xMON-ØØ6Ø3-6)**	Insect resistance (Coleopteran insects) Double herbicide tolerance (glufosinate and glyfosate)	**MON863xMON810** **(MON-ØØ863-5 x MON-ØØ81Ø-6)**	Double insect resistance (Lepidopteran and Coleopteran insects)
Bt11xGA21 **(SYN-BTØ11-1xMON-ØØØ21-9)**	Insect resistance (Lepidopteran insects) Double herbicide tolerance (glufosinate and glyfosate)	**MON863xNK603** **(MON-ØØ863-5 x MON-ØØ6Ø3-6)**	Insect resistance (Coleopteran insects) Herbicide tolerance (glyfosate)

Transformation event (Unique identifier)	Trait	Transformation event (Unique identifier)	Trait
MON88017xMON810 (MON-88Ø17-3xMON-ØØ81Ø-6)	Double insect resistance (Lepidopteran and Coleopteran insects) Herbicide tolerance (glyfosate)	MON89034xNK603 (MON-89Ø34-3x MON-ØØ6Ø3-6)	Insect resistance (Lepidopteran) Herbicide tolerance (glyfosate)
DAS1507xDAS59122 (DAS-Ø15Ø7x DAS-59122-7)	Double insect resistance (Lepidopteran and Coleopteran insects) Herbicide tolerance (glufosinate)	MON89034xMON88017 (MON-89Ø34-3x MON-88Ø17-3)	Double insect resistance (Lepidopteran and Coleopteran insects) Herbicide tolerance (glyfosate)
MON863xMON810XNK 603 (MON-ØØ863-5xMON-ØØ81Ø-6xMON-ØØ6Ø3-6)	Double insect resistance (Lepidopteran and Coleopteran insects) Herbicide tolerance (glyfosate)	DAS59122xDAS1507xN K603 (DAS-59122-7xDAS-Ø15Ø7xMON-ØØ6Ø3-6)	Double insect resistance (Lepidopteran and Coleopteran insects) Double herbicide tolerance (glyfosate and glufosinate)
GA21xMON810 (MON-ØØØ21-9 x MON-ØØ81Ø-6)	*Insect resistance (Lepidopteran insects) Herbicide tolerance (glyfosate)*		
Cotton single events			
MON1445 (MON-Ø1445-2)	Herbicide tolerance (glyfosate)	MON15985 (MON-15985-7)	Insect resistance (Lepidopteran insects)
MON531 (MON-ØØ531-6)	Insect resistance	LLcotton25 (ACS-GHØØ1-3)	Herbicide tolerance (glufosinate)
GHB614 (BCS-GHØØ2-5)	Herbicide tolerance (glyfosate)		
Cotton stacked events			
MON15985xMON1445 (MON-15985-7 x MON-Ø1445-2)	Insect resistance (Lepidopteran insects) Herbicide tolerance (glyfosate)	MON531xMON1445 (MON-ØØ531-6 x MON-Ø1445-2)	Insect resistance Herbicide tolerance (glyfosate)
281-24-236/3006-210-23 (DAS-24236-5 x DAS-21Ø23-5)	*Insect resistance (Lepidopteran insects) Herbicide tolerance (glufosinate)*		
Oilseed rape single events			
GT73 (MON-ØØØ73-7)	Herbicide tolerance (glyfosate)	T45 (ACS-BNØØ8-2)	Herbicide tolerance (glufosinate)
Ms8, Rf3, MS8xRf3 (ACS-BNØØ5-8ACS-BNØØ3-6ACS-BNØØ5-8 x ACS-BN003-6)	Herbicide tolerance (glufosinate) Fertility restoration	*Ms1, Rf1, Ms1xRf1 (ACS-BNØØ4-7 ACS-BNØØ1-4 ACS-BNØØ4-7xACS-BNØØ1-4)*	Herbicide tolerance (glufosinate) Fertility restoration

Transformation event (Unique identifier)	Trait	Transformation event (Unique identifier)	Trait
Ms1, Rf2, Ms1xRf2 (ACS-BNØØ4-7 ACS-BNØØ2-5 ACS-BNØØ4-7xACS-BNØØ2-5)	Herbicide tolerance (glufosinate) Fertility restoration	*Topas 19/2 (ACS-BNØØ7-1)*	Herbicide tolerance (glufosinate)
Soybean single events			
GTS40-3-2 (MON-Ø4Ø32-6)	Herbicide tolerance (glyfosate)	**A2704-12 (ACS-GMØØ5-3)**	Herbicide tolerance (glufosinate)
MON89788 (MON-89788-1)	Herbicide tolerance (glyfosate)	*356043 (DP-356043-5)*	*Double herbicide tolerance (glyfosate and ALS-inhibiting herbicides)*
305423 (DP-305423-1)	*High oleic acid content*	*A5547-127 (ACS-GM006-4)*	*Herbicide tolerance (glufosinate)*
MON87701 (MON-877Ø1-2)	*Insect resistance (Lepidopteran insects)*		
Potato single events			
EH92-527-1 (BPS-25271-9)	Low amylase content		
Sugar beet single events			
H7-1 (KM-ØØØ71-4)	Herbicide tolerance (glyfosate)		
Rice single events			
LLrice62 (ACS-OSØØ2-5)	*Herbicide tolerance (glufosinate)*		

Table 1. GM events authorised in the EU and events under under EC/619/2011 (*in italic*).

A labelling threshold of 0,9% is set up for all authorised GM events in the EU. Food and feed products containing GM events above this threshold have to be labelled as 'containing GMO'. The existence of a labelling threshold requires development of a system for GMO detection and quantification. Several types of methods exist, primarily bioassays, both protein-based (immunological) and DNA-based (mainly based on the Polymerase Chain Reaction (PCR) technology). The protein assays are based on the immunological reaction between the target protein and the specific antibody coupled with colorimetric detection (Holst-Jensen, 2009). Practical applications are the ELISA test or flow strip tests, which are widely used in testing of seed or grain materials. For instance, the United States Department of Agriculture- Grain Inspection, Packers and Stockyards (USDA-GIPSA, 2011) has certified several protein-based rapid kits for detection of biotech-derived grain/oilseeds. However, sensitivity and reliable quantification are often a problem for the immunological assays, due to for example low protein expression. Additionally, proteins are instable and nearly impossible to be reliably detected in processed products. Therefore, the DNA-based methods provide a reliable alternative for detection. In the European Union (EU), the detection of GMO is based on DNA and the recommended technique is real-time PCR. Moreover, this technique also provides the possibility for quantification of the GM target. In this context it is recommended to express the GM percentage as a ratio between the GM

copy numbers and taxon-specific copy numbers (Commission Recommendation EC/787/2004).

The GMO detection policy in the EU is based on two important elements: availability of validated methods for detection and availability of Certified Reference Materials (CRM – see glossary). According to the EU legislation before a new GMO is approved to be released on the market a validated event-specific detection method should be available. The event-specific methods are developed by the company submitting the GMO for authorisation. The company has to develop a method complying with the acceptance criteria described in the document "Definition of Minimum Performance Requirements for analytical methods of GM testing" (ENGL, 2008) developed by the European Network of GMO Laboratories (ENGL – see glossary). The ENGL is a consortium of National Reference Laboratories (NRL – see glossary) assisting the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF – see glossary) by providing scientific expertise. The EU-RL is responsible for testing and validation of the method submitted by the applicant. Upon validation the method is published on the EU-RL web site (http://gmo-crl.jrc.ec.europa.eu/) and made available for further use in the control laboratories involved in GMO testing.

In addition to detection methods, the EU legislation requires availability of Certified Reference Materials for the authorised events (EC/641/2004; EC/1829/2003). The CRM for GM testing are produced by the EC-JRC Institute for Reference Materials and Measurements (IRMM, BE) and the American Oil Chemists' Society (AOCS, USA) and usually are powder or leaf DNA extract with a certified content of the GM event.

The GM testing laboratories have to verify that they are capable to achieve the method performance characteristics before using it for routine analyses by performing in house validation by testing the relevant validation parameters as described in the guidance document (ENGL, 2011). Additionally, the control laboratories must be accredited under ISO 17025 (2005) or another equivalent international standard (Commission Regulation EC/1981/2006).

Although the EU legislation regulates the availability of event-specific methods for GMO detection, other methods such as construct-specific (recognising the GM constructs with which several events are transformed) or element-specific (detecting the elements present in many GMO) methods are used in the control laboratories in order to perform the analysis. These methods are subject to development and introduction of the laboratories themselves: there are no official guidelines describing how to validate such methods and which parameters have to be assessed.

The increasing GM cultivation worldwide and the number of authorisations in the EU and elsewhere pose a significant challenge to the control laboratories. They have to be able to apply all official methods for GM detection of authorised events. A second problem, are the asynchronous approvals of GM events in the EU and third party countries which can lead to low level presence of non-authorised GMO in food and feed. The recently adopted Commission Regulation EC/619/2011 regulates the presence of events which are pending for authorisation or withdrawn from the market in feed and for which methods for detection and reference materials (RM – see glossary) are available (table 1).

Given the fact that an increasing number of events have to be analysed in order to comply with the legislation requirements, the control laboratories need to develop analytical approaches (platforms) which allow them to perform the analyses in a fast, cost and time-efficient manner.

2. Plant DNA extraction and its impact on GMO detection

2.1 Introduction

In view of the EU legislation on GMO commercialisation and the fact that GM events are being authorised, it is mandatory to have control on the products being used and brought onto the market in the EU. Hereto, detection of GM events in food and feed samples is necessary to decide on the conformity of a sample. To enable this detection, real-time PCR (qPCR) is to date the method of choice. For this purpose, DNA needs to be extracted from the sample under analysis. In this process it is important to obtain not only enough DNA to perform the necessary qPCR reaction(s) (part 3) but also DNA of high quality (i.e. purity and integrity). As PCR is an enzymatic reaction, it is kinetically sensitive and the presence of other substances in the reaction may affect the PCR efficiency by for example impairing the binding of the primers to the target sequence in the genomic DNA. Such interference can have an impact on the GMO analysis cascade, especially on the last step namely the GMO quantification.

It has indeed been shown (Corbisier et al., 2007) that the quality of the DNA used in the qPCR has an important influence on the GM% obtained. Depending on the DNA extraction method used and the degree of purity of the extracted genomic DNA (gDNA), a deviating GM% was recorded. An interlaboratory study designed for the maize event MON 810, further demonstrated a significant influence of the DNA extraction method on the measurement results when using the construct-specific qPCR method while this impact was not seen when the event-specific detection method was utilised (Charels et al., 2007). It must thus be noted that even using 'pure' materials such as reference materials, DNA extraction is not so straightforward and that attention should be paid to the choice of the applied extraction method. This becomes even more important for enforcement laboratories as they mainly have to deal with processed and mixed samples. In this respect, Peano et al. (2004) reported the effect of treatment (mechanical, technological, chemical) of a sample in combination with the applied extraction method on the quality of the gDNA. When the feed and food product showed extensive fragmentation, due to a certain treatment during the preparation, the detection of these DNA fragments was dependant on the kit used for DNA extraction. Furthermore, Bellocchi et al. (2010) demonstrated that the result of a quantification experiment may be affected by the DNA extraction method employed unless DNA extracts that do not comply with previously set criteria were removed from the GM% calculations.

This highlights the importance of taking into account different parameters when using a modular approach (Holst-Jensen & Berdal, 2004). It is necessary to set up criteria for DNA quantity, purity, integrity and inhibition prior to using the extracted DNA in the qPCR reactions and to choose an appropriate DNA extraction method. Furthermore, attention should be paid to the fact that different targets might not be affected in the same way by impurities or co-extracted substances. Both Corbisier et al. (2007) and Cankar et al. (2006) demonstrated that this would impair in a strong way the final result. If, in a GMO quantification the two targets (i.e. the transgene and the taxon-specific element) do not

behave in the same way and the PCR efficiencies are deviating too much, the obtained GM% would be biased.

It should also be noted that the extraction method used has a double impact on GMO quantification as not only the sample needs to be extracted but also the CRM. As the DNA extracted from the CRM powder will be used to construct the calibration curve in the quantification experiment it should also be free of inhibitors as this otherwise will affect the PCR efficiency. DNA extracted from the CRM powder needs to be pure and free of inhibitors to obtain a curve falling within the ENGL criteria (ENGL, 2011). Additionally, the PCR efficiencies for the calibrant and the sample should be the same to obtain reliable quantification. As this is not always the case, controls such as dilutions of the sample to evaluate inhibition, should be included in the reaction (point 2.2).

Although many DNA extraction protocols are quite user friendly and many extraction kits exist, their downstream application in qPCR is not clear-cut and additional evaluation of the quality of the extracted gDNA is necessary as well as assessment of the presence of possible PCR inhibitors.

2.2 Assessment of DNA yield, purity, integrity and inhibition

The determination of the DNA concentration in an extract is not straightforward and different techniques exist. The obtained **DNA yield** after extraction can, for example, be determined using spectrophotometry (UV). This determination is based on the absorbance of nucleic acids at a wavelength of 260 nm. It is a method that has been used commonly for the estimation of the concentration of nucleic acids in a range of applications (Sambrook & Russell, 2001). Although it is a fast and simple method, it allows only determination of the concentration in a range of 5 to 50 µg/ml. Another drawback of this method is the fact that it is not specific for double stranded DNA (dsDNA) but also detects RNA and single stranded DNA (ssDNA) molecules (Gallagher, 2011). Additionally, substances like proteins and phenolics also absorb between 220 and 340 nm and can thus interfere with the measurement.

Alternatively, fluorimetry can be used to determine the concentration of the extracted gDNA in the solution (Singer et al., 1997). This method uses a dye that fluoresces upon intercalating in the dsDNA such as the PicoGreen (Molecular Probes). This enables a more specific measurement of the dsDNA amount present in an extract as there is no binding with interfering proteins and only a limited interaction with RNA and ssDNA. This method is more sensitive than UV measurements permitting to work with samples with lower concentrations in a linear range of 0,05 to 1 µg/ml (Singer et al., 1997). The method is reliable and well introduced in GMO testing laboratories. It should however be noted that a standard curve using lambda DNA needs to be prepared which requests a little more time. Furthermore it has been observed that the presence of various compounds have an effect on the accuracy of PicoGreen-based measurements (Singer et al., 1997; Holden et al., 2009 – see below).

A deviation between the concentration obtained by UV measurement and fluorimetry is often seen (Holden et al., 2009), especially for highly processed products (Bellocchi et al., 2010). This may be due to the fact that short or single stranded nucleic acid fragments interfere more with UV than with the PicoGreen dye. It has been proven that the fluorescence signal decreases with increasing length of sonication time (and thus fragmentation) showing the inability of the PicoGreen dye to bind with single stranded

fragments (Georgiou & Papapostolou 2006; Holden et al., 2009; Shokere et al., 2009). One of the possible sources of single-stranded DNA may be denaturation of DNA during the drying phase after ethanol precipitation, the final step in many extraction protocols (Svaren et al., 1996). Utilizing spectrophotometry to quantify the DNA in an extract may thus lead to overestimation of the concentration.

Although one should determine the concentration of an extract to ensure that the DNA amount in a quantification reaction is above the limit of quantification (LOQ –part 5), the exact DNA concentration is of less importance. As the determination of the GM content of a sample relies on a relative calculation (ratio transgene copies versus endogene copies – part 3), it is imperative that a same amount of DNA is engaged in both qPCR reactions necessary in quantification, i.e. the event-specific and taxon-specific qPCR methods, whereas the exact amount engaged is of lesser importance. Carrying out both reactions in a single well, i.e. performing a duplex reaction would thus be a good solution.

When using spectrophotometry, additional to measurements at 260 nm, also measurements at wavelengths of 230 and 280 nm may be done. The **purity** of the DNA can then be assessed using the absorbance ratios A260/280 and A260/230. The A260/280 ratio gives an idea of the occurrence of residual proteins. On the other hand, the A260/230 ratio gives an indication on the presence of carbohydrates. In an ideal situation, both ratios should tend to 2,0 (Glasel 1995; Manchester 1995). Any deviation could indicate the presence of co-extracted materials that can impair the availability of the DNA for hybridisation with the primers and thus affect the PCR efficiency.

Another important aspect is the **integrity** or intactness of the gDNA (degradation). When the DNA becomes fragmented, the GM target which is less abundant (compared to the endogene) might fall below the quantification limit of the qPCR method. It is evident that this has a practical consequence on the correct quantification of the target. One must thus ensure that the average length of the extracted DNA molecules is longer than the size of the amplicon. To avoid that degradation of the DNA impairs the GMO quantification, the methods are generally designed to amplify sequences ranging in size between 70 and 100 bp. However, one should take into account the minimum length of an amplicon necessary to allow binding of the oligonucleotides (two primers in SYBR®Green chemistry, two primers and one probe used in TaqMan® chemistry). To this purpose for example MGB probes (Kutyavin et al., 2000) can be used to allow even shorter sequences that are stable and have an elevated melting temperature. Further, the amplicon sizes for the endogene and transgene target should not differ too much as shorter fragments are more efficiently amplified than longer ones. This difference in amplification efficiencies will have an impact on the correctness of the quantification reaction. The intactness of the extracted DNA can be assessed using agarose gel electrophoresis with ethidium bromide staining or an alternative. This technique also allows observing if any RNA has been co-extracted.

Knowledge of the presence of co-extracted substances and RNA and the existence of fragmented DNA in the extract is however not sufficient. It is known that **PCR inhibitors** are one of the most important influencing factors of the reliability of quantification (Bickley & Hopkins, 1999). It is thus important to know the impact of these molecules, present in the solution, on the GM quantification. Hereto, a preliminary inhibition test should be performed to evaluate their possible effect on the PCR efficiency. In this view, it is important to check if both targets of the quantification reaction (i.e. endogene and transgene) are equally affected by

the presence of the inhibitors. If this is not the case, it would influence the detection of the real number of targets and thus lead to a deviating result (Corbisier et al., 2007).

There are several ways to study the presence of inhibition in a qPCR reaction. It is for example possible to include Internal Amplification Controls (IAC; Nolan et al., 2006; Burggraf & Olgemoller, 2004) or to add a positive control nucleic acid to the sample (Cloud et al., 2003). Further, mathematical algorithms can provide a measure of PCR efficiency from analysis of the amplification curves (Tichopad et al., 2003; Ramakers et al., 2003; Liu and Saint, 2003; Lievens et al., 2011). A simple alternative is the use of dilution series to assess the impact of inhibitory substances on the PCR reaction.

Recently, the ENGL released a document wherein they describe an approach to evaluate inhibition of a PCR reaction (ENGL, 2011). To this purpose the gDNA is serially diluted and each dilution is measured in duplicate using the validated qPCR method that will be applied for quantification. According to the previously published ENGL document (2008), the difference between the measured and theoretical C_t value should not exceed 0,5 C_t to exclude inhibition. In practice, four four-fold dilutions (from 1/4 till 1/256) need to be prepared from a stock solution. Both the dilutions and the stock are subsequently analysed in qPCR. This yields five qPCR results: the undiluted sample and the four (four-fold) dilutions. Using the latter, a curve is constructed by regressing the C_t values against the log of the dilution factor. This relation then allows the calculation (extrapolation) of a theoretical C_t value for the undiluted sample. Subsequently, this 'extrapolated' C_t value is compared with the measured value: there should be no more than 0,5 difference. Additionally, the regression line should comply with the following criteria: the slope must be between -3,6 and -3,1 and the linearity (R^2) must be equal or above 0,98.

A practical adaptation of this method is being used in the WIV-ISP-GMOlab. A series of dilutions is made from the gDNA under investigation and each dilution is analyzed using qPCR. Subsequently it is assumed that the last dilution contains the least inhibitors as the co-extracted substance will be diluted together with the DNA and will be below inhibitory concentration. The theoretical/expected C_t can be calculated for the other dilutions using knowledge of the dilution factors (e.g. a dilution of 2 corresponds to a C_t difference of 1). If the difference between the measured and theoretical C_t is equal or below 0,5, inhibition can be excluded. It must be noted that a difference of 0,5 for the highest concentration can be considered as an indication of inhibition. If this is observed for lower concentrations (more diluted samples) it is more probable that it comes from a dilution or pipeting mistake as it is unlikely that a low concentration would show inhibition that is not seen in the more concentrated solution.

These experiments and criteria should be set up by the laboratories prior to the quantification qPCR reaction to ensure correct quantification of a GM event in a sample. It should hereby be noted that also the DNA extracted from the CRM, used to construct the calibration curve, should be subjected to an inhibition test. Furthermore, these criteria should be evaluated for each DNA extraction method in combination with at least the most common matrices.

2.3 Evaluation of DNA extraction methods

Samples under investigation in GMO detection can vary to a great extend in the context of composition (single ingredient versus mixture), texture (solid versus liquid) and matrix

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(different plant species, processed versus raw material). The use of one universal DNA extraction method can thus difficultly be envisaged. The choice of an appropriate extraction procedure suitable for a particular sample matrix is thus a prerequisite for successful qPCR analysis. It must however be noted that this is not always straightforward as enforcement laboratories are not necessarily informed on the ingredients present in the sample under investigation.

The C-hexadecyl-Trimethyl-Ammonium-Bromide ('CTAB') extraction method is widely used in the enforcement laboratories for GMO detection (Pietsch et al., 1997). The method starts with lysis of the cells to release all contents. Addition of RNase and Proteinase K allows removal of respectively RNA and proteins. The ionic detergent CTAB forms an insoluble complex with the nucleic acids. The polyphenolic compounds, polysaccharides and other components remain in the supernatant and can be washed away. The DNA is released from the pellet by raising the salt content and is then concentrated by alcohol precipitation. It can be used for a variety of matrices such as maize, oilseed rape, potato and rice. The DNA yield is in most cases sufficient to conduct the necessary qPCR steps. However, the purity of the DNA solution is not always satisfactory. Yet, it is one of the more suitable methods for processed food and feed. In any case, an inhibition test is always advisable. In the GMOlab, inhibition is sometimes seen with very complex matrices such as processed feed products and liquid samples. The protocol is also less efficient for some rice containing materials. One of the drawbacks of the CTAB method is that the procedure is quite time-consuming as it contains different steps of incubation and centrifugation and also an overnight step necessary to ensure that the DNA pellet is completely dissolved. The method further requires some pre-extraction manipulations such as the preparation of specific buffers. It should also be noted that residues of the CTAB buffer can interfere with the PicoGreen dye and impair a correct measurement of the DNA concentration. It was observed that the magnitude of the effect of the CTAB detergent was in inverse proportion to the amount of DNA in the assay (Holden et al., 2009).

The CTAB extraction method can alternatively be combined with an extra purification step. Hereto a Genomic-Tip 20 column can be used (QIAGEN). This is an anion-exchange chromatography column to which the DNA fragments will be bound by electrostatic interactions between the negatively charged phosphate groups of the DNA and the positively charged resin. Upon subsequent washing steps, the impurities are removed while the DNA remains bound to the column. Finally the DNA is eluted and precipitated with alcohol. The method is very efficient for DNA extraction from soybean and cotton matrices which are more difficult to extract using the classic CTAB extraction method. For cotton powders for example, this is also the method recommended by the EU-RL (http://gmo-crl.jrc.ec.europa.eu/summaries/281-3006%20Cotton_DNAExtr.pdf). Utilizing this alternative procedure, solutions of higher purity can be obtained although the DNA yields are lower. However, they are in most cases still sufficient to perform all necessary qPCR analyses. Due to the purification of the gDNA on the column, these extracts are most often free of inhibitors. As for the classic CTAB method, specific buffers need to be made and an overnight step has to be incorporated to allow the pellet to dissolve. Additionally, the Genomic-Tip 20 columns and buffers that need to be purchased tend to be rather expensive.

A big advantage of the CTAB and CTAB-Tip20 methods is that there is no restriction on the sample intake. This allows the laboratories to easily scale up the extraction protocol. This is for example very convenient for the extraction of gDNA from CRM to ensure sufficient

DNA for validation of methods. The production of large batches of CRM DNA allows the laboratory to have a tested material readily available for several subsequent experiments. Also for several samples such a scaling up is sometimes necessary as the DNA content of some samples may be very low (due to for instance processing).

To reduce the time of DNA extraction, several kits are commercially available. Different companies offer their own DNA extraction kit which is mostly based on isolation of the gDNA using a silica-based method. Usually these kits deliver very fast gDNA and are easy to handle. A drawback of these kits is that often the sample intake is limited which has an impact on the final DNA yield. In, for example, the Wizard Genomic DNA Purification Kit (Promega), a maximum intake of 20 mg is allowed. It is thus necessary to pool several extracts to obtain a sufficient DNA amount for the subsequent qPCR analysis. In addition, when using DNA extracted with this kit, fluctuations in PCR efficiencies upon repetitions were observed which could lead to over- or underestimation of the GMO content (Cankar et al., 2006). Moreover, when comparing the PCR efficiencies of different amplicons, the gDNA extracted with the Wizard kit showed a high dispersion of the data.

The GENESpin kit (Eurofins GeneScan) is one of the few kits where an indication for possible scaling up of the system is given. According to the manufacturers, the kit would be suitable for several food samples such as cakes, bread, sausages,… They also indicate adapted protocols for liquid and powdered hygroscopic samples.

Furthermore, it should be noted that the kits are not always suitable for the extraction of DNA from all matrices. The DNeasy plant kits (QIAGEN) for example, are very efficient kits for the purification of DNA from fresh material (leaves, roots,…) but are less suited for powder materials. Corbisier et al. (2007) showed in their pilot study that this kit yielded a DNA concentration that was twice as low in comparison to the CTAB method. However, using this protocol relatively pure extracts were obtained. In the same study, it was observed that the Nippon Gene GM Quicker protocol (Diagenode), although a low yield and purity was achieved, delivered DNA which was less contaminated by RNA in comparison to the other procedures used.

The situation is even more complicated when it comes to DNA extraction of real-life samples. These not only can contain different species but also additional substances that affect DNA extraction. One such example is the presence of lecithin. This substance is often used in bakery products and as emulgator, stabilisator or anti-oxidant. Additionally, some products such as soybeans contain natural lecithin. As soybean is widely used in food and feed materials and Roundup Ready Soybean is one of the most cultivated GM crops (James, 2010), GMO detection laboratories often have to deal with this product. Wurz et al. (1998) presented an efficient extraction protocol for the isolation of soybean DNA from soy lecithin and showed its application in downstream qPCR. This method can thus be used for extraction of DNA from products such as soymilk and soy sauce.

Last but not least, it should be taken into account that the same product (e.g. bread) can have a different composition when produced by different procedures and can thus contain different substances that could affect the efficiency of the PCR. Even when taking for example only soybean products into account, the PCR efficiency is very much dependant on the nature of the product (Cankar et al., 2006). It was reported that for example DNA extracted with the DNeasy kit (QIAGEN) from a soybean feed sample revealed a higher

inhibition effect on the transgene compared to the endogene although that for other samples such as the CRM, soybean milk and tortilla chips this was not observed.

It is thus advisable to validate an extraction method for different matrices. And although the extraction method is validated for a certain matrix, one should keep in mind that gDNA extracted from different samples is not necessarily equally suitable for quantitative analysis. Considering this, it is worthwhile for a GM detection laboratory to put some effort in the evaluation of the different existing extraction protocols in combination with the variety of samples that need to be analysed in GMO detection. And subsequently to chose the extraction method that is the most suitable to remove potential compounds such as lipids, polysaccharides and phenolics that could otherwise impair the PCR efficiency.

2.4 Conclusion

GM quantification is performed in different steps in which DNA extraction is the first one. This pre-PCR phase is of great importance for the trueness of the quantification result. The DNA extracted from different materials should be evaluated for yield, purity and integrity before performing the qPCR experiment. Furthermore, the DNA solution should be assessed for the presence of inhibitors and their impact on the two targets of the quantification i.e. the endogene and transgene. It is clear that these parameters not only have to be evaluated for the sample under investigation but also for the gDNA extracted from the Certified Reference Material used as a calibrant. Both the sample and CRM DNA need to meet the set criteria to ensure reliable quantification. Seen the diversity of products and matrices that need to be analysed by GM testing laboratories, several DNA extraction protocols exist including home-made buffers and kits. It is obvious, that the extraction protocol to be used needs to be evaluated and that the gDNA extracted has to pass the requirements set by the laboratories before it is used in subsequent PCR analysis. In addition to the choice of the DNA extraction method, thought should also be given to the method used to determine the concentration of the extracted DNA.

In general, the validated DNA extraction protocols used in routine such as the CTAB method are valid for different matrices. However, when dealing with a complex matrix it is important to verify the quality of the DNA. As the extraction method may in some cases have an influence on the GM content, optimalisation of the extraction procedure may be needed. Furthermore, the presence of inhibitors should be checked as they may impair the efficiency of the PCR reaction and thus influence the quantification of GM events in a sample. Hereto, the impact of co-extracted substances and products used in the extraction protocol should be evaluated on the sample, the CRM and the two targets under investigation. If a considerable inhibitory effect is observed, further DNA purification should be performed.

3. Description of the structure of a transgenic insert and the type of DNA sequence used for qPCR analysis

3.1 Introduction

All the GM events currently on the EU market are plants in which a piece of foreign DNA has been introduced into the genome. This piece of DNA generally consists of a regulatory promoter region, a coding sequence and a terminator (Fig. 1) and is called the transgenic construct or insert. To introduce this construct into the plant genome, genetic engineering

techniques (Darbani et al., 2008), such as *Agrobacterium*-mediated transformation and particle bombardment, are being used. Hereto the transgene is cloned in a plasmid for example between two specific and unique sequences (T-DNA borders).

For *Agrobacterium*-mediated transformation, the plasmid carrying the transgene is introduced into this bacterium. Further, the intrinsic properties of this soil bacterium are used to incorporate the transgenic construct into the plant genome: the bacterium namely infects the plant and transfers the T-DNA part of the plasmid to the plant genome. In this way the transgene is stably inherited in the subsequent generations (Chilton et al., 1977). Different explants such as leaves (Horsch et al., 1985), roots (Valvekens et al., 1988), embryos (Hensel et al., 2009), ovules (Holme et al., 2006) and microspores (Kumlehn et al., 2006) can be used for transformation. In particle bombardment, gold or tungsten particles are coated with the plasmid containing the transgene (Kikkert et al., 2004). Subsequently, these particles are fired onto the explants with high voltage allowing the incorporation of the transgene into the plant genome. Compared to *Agrobacterium*-mediated transformation, particle bombardment more often leads to multiple inserts of the transgenic construct into the genome.

The detection of this transgenic insert forms the basis of the EU legislation concerning the introduction of GMO onto the market and thus requests the development of GMO detection methods. This detection is carried out by enforcement laboratories and the method of choice is real-time PCR (qPCR). At WIV-ISP, a GMO detection platform, allowing the verification of the presence of GM material in food and feed samples was developed. The platform consists of
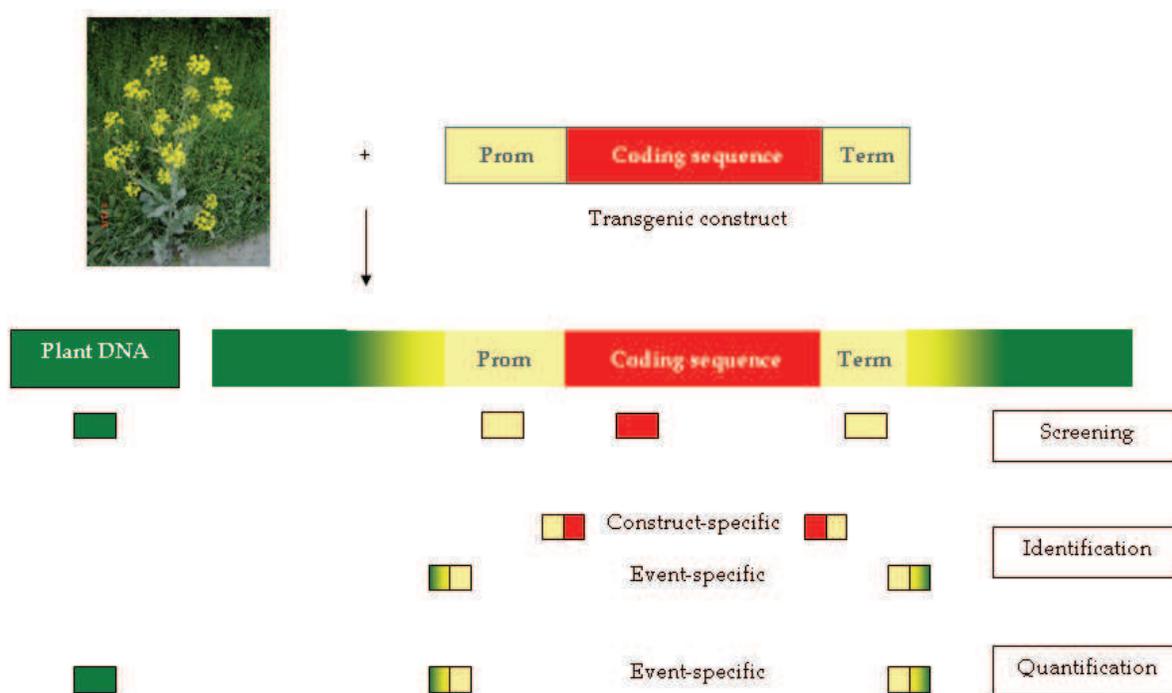

Fig. 1. Plant transformation and type of sequence targeted by the different steps in qPCR analysis.

In screening, a sequence inside one of the elements of the transgenic construct is targeted. A construct-specific method used for the identification of the GMO targets the junction between two elements within the transgenic construct. An event-specific method, used in identification and quantification of a GM event, targets the junction between the transgenic insert and the plant genome DNA.

a preparative step namely DNA extraction (part 2) and three consequent qPCR steps namely screening, identification and quantification (Fig. 2). Hereto, in-house developed and validated SYBR®Green screening methods (part 4) are combined with EU-RL validated TaqMan® event-specific methods (part 5). In each step of the qPCR analysis, a different part of the transgenic construct is being targeted. The region in the construct targeted by the method is linked with the specificity of the method. By using a more specific method in each subsequent step, it is possible to gradually narrow down the possibilities to a specific GM event.

Homogenisation	Grinding, mixing, shaking
DNA extraction	CTAB, CTAB-Tip20, extraction kits
DNA quantity and quality	DNA concentration, purity, integrity, inhibition test
qPCR Screening	SYBR®Green element-spec method
qPCR Identification	TaqMan event-spec method
qPCR Quantification	TaqMan event-spec methods Transgene & Endogene
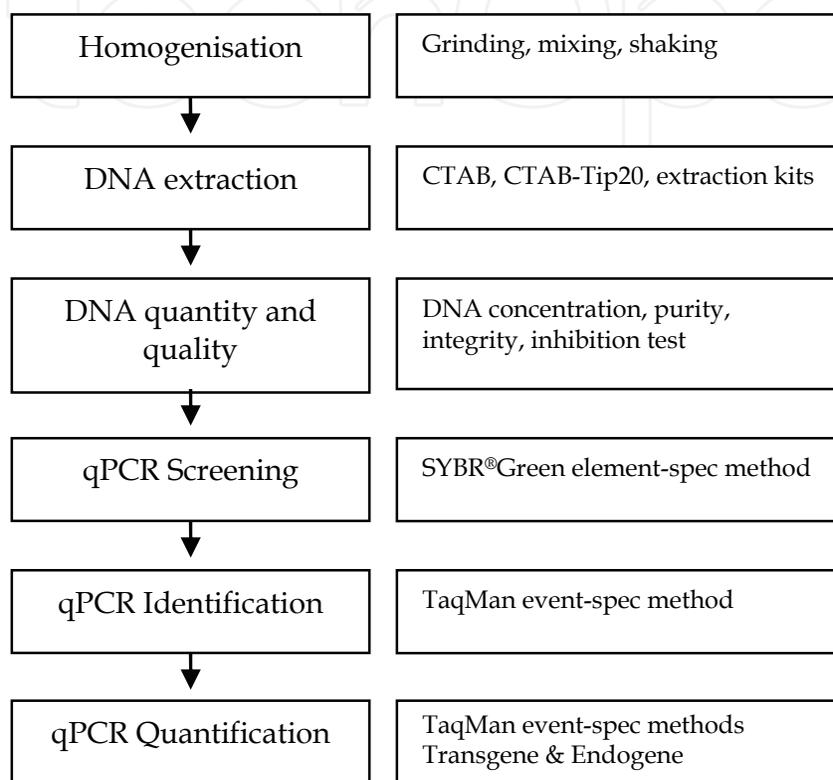
Fig. 2. Flowchart of the analysis steps in GMO detection

In support of these analyses, a matrix-based approach called CoSYPS (Combinatory SYBR®Green qPCR Screening) has been developed (Van den Bulcke et al., 2010). This approach relies on the integration of the analytical results obtained for a sample in a mathematical Decision Support System and the application of a "prime-number"-based algorithm (part 6). Based on the outcome of the screening results of a set of markers in a sample, the system will identify which GM events are possibly present in a sample.

3.2 GMO screening methods

After DNA extraction, screening is the next crucial step in GMO detection. In view of the growing number of GM events introduced on the market and new upcoming traits, screening methods will become more and more important and necessary to enable the discrimination between the different GMO. Testing for each possible GM event separately would namely become too expensive and labour-intensive.

A screening method usually targets a sequence inside one of the elements of the transgenic construct (Fig. 1). Seen the fact that the elements that are used in transgenic constructs are

recurrent, detection of a single element often does not confer high specificity and, as a consequence, does not allow deciding on which GM event might be present. A combination of different screening markers is therefore necessary to get a better idea of the possible GM events occurring in a sample. This allows the reduction of the number of identifications to be performed.

To date several screening methods for the detection of GM materials in food and feed samples have already been published. These methods often target the Cauliflower Mosaic Virus 35S promoter (p35S) and/or the *Agrobacterium tumefaciens* nopaline synthase terminator (tNOS) seen the fact that these elements are the most represented in the EU authorised GM events. From the twenty four authorised events, nineteen events contain the p35S target, fifteen the tNOS element and eleven combine both markers (GMO Compass website; Agbios website). Additionally, methods for the detection of herbicide tolerance (HT) genes used in transgenic constructs have been reported. These mainly target two classes of HT sequences: the bacterial phosphinotricin-*N*-acetyltransferases from *Streptomyces viridochromogenes* (*pat*) and from *Streptomyces hygroscopicus* (*bar*) (Wehrmann et al., 1996), and the 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) from *Agrobacterium tumefaciens* strain CP4 or from plant origin (*in casu* petunia) (Kishore et al., 1988; Padgette et al., 1996). Apart from herbicide tolerance, the GM events currently on the market are transformed with insect resistance traits. Hereto the *Bacillus thuringiensis* endotoxin encoding genes (e.g. the *cryIAb/Ac*) are being used and detection methods have been developed (Bravo et al., 2007). It should however be noted that the above-mentioned methods are mostly either end-point detection on agarose gel or real-time qPCR using TaqMan® chemistry (Hamels et al., 2009; Raymond et al., 2010; Nadal et al., 2009; Prins et al., 2008). Development of screening methods using the SYBR®Green qPCR technology only started recently (Barbau-Piednoir et al., 2010; Barbau-Piednoir et al., 2011; Mbongolo Mbella et al., 2011) although this approach offers a number of advantages over the TaqMan chemistry. The use of melting temperature analysis for instance allows detection of the expected target but also allows distinction between closely-related elements, which is important in the evaluation of the specificity of the method. But more important for enforcement laboratories is the fact that SYBR®Green methods do not require the use of fluorescent labelled oligonucleotides which is much more cost effective.

In view of the growing amount of GM events and the lack of cost-effective screening methods, the WIV-ISP platform puts a major effort in the development of an extensive number of qPCR SYBR®Green screening methods. They form a unique combination targeting different elements within the transgenic construct in addition to plant sequences and are gathered in the patented CoSYPS matrix (Combinatory SYBR®Green qPCR Screening; Van den Bulcke et al., 2010). The methods used to build the CoSYPS were in-house developed and validated (part 4). They are used together with the CoSYPS matrix in the routine analysis of food and feed samples in the GMOlab under ISO 17025 accreditation. To cover the increasing number of GM events and to add discriminative power to the CoSYPS system, new screening methods are being developed on a regular basis and are subsequently being introduced in the CoSYPS (part 6) after in-house validation.

The in-house developed methods target different types of DNA elements (table 2). Firstly, a screening method aiming to target the chloroplastic *rbcl* gene (plant kingdom marker) was developed. This element will permit to decide on the presence of vegetative DNA in an unknown sample. Secondly, methods that detect plant taxon-specific sequences (Mbongolo

Method name	Target	Fragment size (bp)	Reference
Plant kingdom marker			
Rbcl	Ribulose-1,5-biphosphate carboxylase oxygenase	95	Mbongolo Mbella et al., 2011
Plant taxon-specific methods			
Lectin	Lectin gene of soybean (*Glycine max* L.)	81	Mbongolo Mbella et al., 2011
Adh	Alcohol dehydrogenase I gene from maize (*Zea mays* L.)	83	Mbongolo Mbella et al., 2011
Cru	Cruciferin gene from oilseed rape (*Brassica napus*)	85	Mbongolo Mbella et al., 2011
PLD	Phospholipase D gene from rice (*Oryza sativa*)	80	Mbongolo Mbella et al., 2011
Sad 1	Stearoyl-acyl carrier protein desaturase gene of cotton (Gossypium genus)	107	Mbongolo Mbella et al., 2011
Glu3	Glutamine synthetase gene from sugar beet (*Beta vulgaris*)	118	Mbongolo Mbella et al., 2011
Methods specific for generic element			
p35S	Promoter of the 35S Cauliflower Mosaic Virus	75	Barbau-Piednoir et al., 2010
tNOS	Terminator of the nopaline synthase gene	69	Barbau-Piednoir et al., 2010
pFMV	Promoter of the 34S Figworth Mosaic Virus	79	Broeders et al., (in preparation)
pNOS	Promoter of the nopaline synthase gene	75	Broeders et al., (in preparation)
t35S	Terminator of the Cauliflower Mosaic Virus	107	Broeders et al., (in preparation)
Methods specific for GM elements			
CryIAb	Gene encoding the *Bacillus thuringiensis* δ-endotoxin (insect resistance)	73	Barbau-Piednoir et al., 2011
Cry3Bb	Gene encoding the *Bacillus thuringiensis* δ-endotoxin (insect resistance)	105	Broeders et al., (personal communication)
Pat	Phosphinotricin-*N*-acetyltransferases gene from *Streptomyces viridochromogenes*	109	Barbau-Piednoir et al., 2011
Bar	Phosphinotricin-*N*-acetyltransferases gene from *Streptomyces hygroscopicus*	69	Barbau-Piednoir et al., 2011
EPSPS-CP4	5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium tumefasciens* strain CP4	108	Barbau-Piednoir et al., 2011
P35S discriminating method			
CRT	Reverse transcriptase gene from the Cauliflower Mosaic Virus	94	Papazova et al., (in preparation)

Table 2. List of SYBR®Green screening methods developed and validated by the GMOlab.

Mbella et al., 2011) have been developed. These methods target the main GM commodity crops such as soybean, maize, oilseed rape, cotton, sugar beet and rice. They make it possible determining the species composition of the sample and allow a first discrimination of GM events (e.g. the presence of a soybean GM event can be excluded if the soybean taxon-specific marker is negative). Thirdly, methods specific for GM generic elements were developed (Barbau-Piednoir et al., 2010). These are elements that are included in many transgenic constructs used in commercial GM plants. Such elements are represented by promoter and terminator sequences such as the Cauliflower Mosaic Virus promoter (p35S) and the *Agrobacterium tumefaciens* nopaline synthase terminator (tNOS). Adding the information from the qPCR experiments targeting these generic elements gives a first idea of the putative presence of a GM event in the sample. However, seen these elements are widespread in the transgenic constructs currently used, they do not contain enough discriminative power to sufficiently reduce the number of possible GM events present. These elements need thus, in a fourth step, to be combined with methods targeting other GM specific elements such as herbicide tolerance and insect resistance genes (e.g. Cry genes, bar, pat). Such methods have also been developed and were recently published (Barbau-Piednoir et al., 2011). Last but not least, a marker was developed to be able to discriminate between the p35S present in a GM event and the one due to possible natural presence of the Cauliflower Mosaic Virus from which the transgenic sequence was originally taken (the so-called donor organism). The combination of the results of the eighteen markers, currently used in routine, will allow defining the putative GM events present in a sample. Utilizing the CoSYPS to this purpose, a list of possible events to be identified will be obtained. Additionally, the use of the various markers in combination with the CoSYPS is a powerful tool in the detection of unauthorised GMO (UGM) events. In principle, the elements that are positive in the screening qPCR should be covered by the EU authorised events (EC/1829/2003) or the GM events included in the 'Low Level Presence' legislation (EC/619/2011). If this is not the case, one might suspect the presence of an unauthorised event in the sample.

For each of the screening methods developed and validated at the WIV-ISP-GMOlab, the corresponding amplicon is cloned in a pUC18 background. These plasmids, called Sybricons, are submitted under "Safe Deposit" at the BCCM (Ghent, BE). They can be used to determine the nominal T_m value of the target and further utilized as positive controls in routine analysis.

In addition to the 18 SYBR®Green screening markers, the GMOlab applies two markers in TaqMan® chemistry for the detection of potato (UGPase) and linseed (SAD).

3.3 GMO identification methods

Based on the outcome of the screening step, a second phase will be necessary namely identification of the GM event.

Identification methods are directed to the detection of a specific GM event. These qPCR methods, contrary to the screening methods, use TaqMan® chemistry. They can be either construct-specific or event-specific qPCR methods. A construct-specific method targets the junction between two elements within the transgenic construct. They are thus directed to the sequence covering a part of the promoter and coding sequence or of the coding sequence and the terminator (Fig. 1). Event-specific methods, in contrast, target the junction between the transgenic insert and the plant genome DNA. They are thus designed to cover part of the sequence of the plant and the promoter or of the terminator and the plant DNA (Fig. 1).

As the location of the transgenic insert into the plant genome is unique, the event-specific methods are specific to a sole GM event. Indeed, one and the same construct can be inserted into the genome of different plant species and will not be discriminated by using a construct-specific method alone whereas the plant-insert junction, targeted by the event-specific method, will be unique. This makes the event-specific methods the technique of choice in GMO identification. These methods are in fact part of the GM quantification methods available. They are laid down by the GM Company together with the request for GM authorisation. Subsequently the EU-RL validates them in a ring trial in which the NRL for GMO detection participate. Once the validated method is published and a CRM is available, the enforcement laboratories need to be able to implement the method in their laboratory (part 5). The construct-specific methods, on the other hand, can be in-house developed methods, methods developed by research groups or the qPCR methods that are published by the EU-RL for quantification of GM events. As they are less specific than the event-specific methods, they have a less discriminative power and are thus not recommended. However, for some GM events (e.g. rice GM events) no other methods exist to date.

At the GMOlab, the coming out of the different identifications are gathered in a Decision Support System (part 6) which will further indicate at which level a specific GM event is present. Only if the GMO is found at quantifiable levels (i.e. above the limit of quantification), a third step will be involved namely quantification of the GM event.

3.4 GMO quantification methods

In this last step in the process of GMO detection, the amount of the present GM event will be determined. This quantification is necessary to assess the compliance of a sample with the 0,9% labelling threshold (EC/1829/2003) and the recently voted 'Low Level Presence' (LLP) legislation (EC/619/2011).

Quantification of a GM event in a sample relies on the relative determination of the number of copies of the transgene in relation to the number of copies of the endogene (i.e. the taxon-specific sequence). Hereto a combination of a GM event-specific method and a taxon-specific method will be used. Both methods need to be provided by the GM plant developing companies when requesting EU authorisation and are subsequently validated by the EU-RL. Each laboratory involved in GMO detection needs then to verify in-house if the method complies with the set acceptance criteria before to use it in routine analysis of samples (part 5).

The result of GMO quantification is expressed as a GM mass percentage in relation to the ingredient for authorised events and in relation to the GM material for the LLP events. This result is reported to the competent authorities who will decide if the sample is conform to the legislations or not.

3.5 Conclusion

As the number of GM events being introduced on the market is rapidly increasing, screening will become a necessary first step in GMO detection. Additionally, an intensive screening provides an indication on the presence of GM material originating from unauthorised and unapproved GMO. Indeed, countries that produce GM plants only for local consumption will not request for EU authorisation but these crops might still "escape" and end up in the EU food chain. As a consequence also the detection of these UGM will become a major task of enforcement laboratories.

The GMO platform developed by the WIV-ISP-GMOlab allows detection of authorised GM events as well as UGM in a cost- and time effective manner. It consists of a preparative DNA extraction step and three consecutive qPCR steps. The CoSYPS system, including in-house developed SYBR®Green screening methods, forms an innovative tool in GMO detection allowing reducing the number of identifications to be carried out. The TaqMan® identification further allows a narrowing down of the GM events present to a specific GMO and quantification permits the determination of the GM content.

4. Development and validation of a qualitative qPCR method in view of its application for screening purposes in the WIV-ISP GMO detection platform

4.1 Introduction

As described previously, in order to face the rapidly increasing number of GMO in food and feed products, new methods facilitating an initial screening of analytical samples is needed. Therefore, one of the major objectives of the molecular platform at WIV-ISP is to develop qualitative screening methods targeting either new genetic elements commonly found in transgenic constructs or species frequently used in food and feed in view of rationalizing GMO detection.

The methods developed are singleplex qPCR, based on SYBR®Green chemistry. Additionally, the methods are designed to work under uniform conditions (primer concentrations, PCR program) in order to facilitate their simultaneous application in a 96-well plate format. These SYBR®Green methods were in-house validated in order to be applied under ISO 17025 accreditation. As there is no 'golden standard' for the validation of qualitative methods related to GMO detection, enforcement laboratories need to decide which parameters need to be evaluated in the validation. In addition, the laboratories have to set their own criteria based on the guidance document for quantitative qPCR methods.

Part 4.3 of this chapter focuses on the method validation criteria and proposes a pragmatic approach for the in-house validation of singleplex real-time PCR qualitative methods. This proposal is mainly based on the recently adopted Codex Alimentarius guidelines on performance criteria and validation of methods for GMO analysis (Codex, 2010), and on the minimum performance requirements for methods for GMO testing set forward by the ENGL (ENGL, 2008). During the in-house validation critical values are determined for the screening methods to be introduced in the Decision Support System currently used in the routine analyses, namely the CoSYPS (part 6).

4.2 Development of SYBR®Green methods for screening purposes

The first step of method development is to determine the screening qPCR target. Targets for screening can be any element present in the transgenic construct inserted in authorised or unauthorised GMO and taxon-specific sequences. Application of the screening approach requires development of many targets in order to cover the growing range of GM events. Selection of the methods to be developed is based on a number of priorities. Firstly, methods targeting the main commodity crops used in transformation events are of high importance. Secondly, priority is given to transgenic elements frequently occurring in EU authorised GM events in addition to targets that provide an extra discriminative power. Thirdly, other

important transgenic elements occurring in unauthorised GM events which might be necessary to test for by the enforcement laboratories should be targeted.

The development of a new screening method depends on several prerequisites: information on the elements inserted in a GM event, their copy number and the nucleotide sequence of the inserted element. Information on the elements of the transgenic construct inserted in a GMO can be obtained from publicly available dossiers submitted by the applicant for authorisation or patent databases. This information is usually available after the authorisation is granted or after the competent authorities have given a positive advice. Important information sources are the GMO crop database of the Centre for Environmental Risk Assessment (CERA) (http://www.cera-gmc.org/?action=gm_crop_database) and the GMO database on authorisations and approval of GMO in the EU (http://www.gmo-compass.org/eng/gmo/db/). The nucleotide sequences are available in public databases such as the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), patent databases and scientific publications. One must however take care when using the information present in these databases as for example Single Nucleotide Polymorphisms (SNP) may exist in the sequence of the elements inserted in different GM events (Morisset et al., 2009). Therefore, the information in the public databases is not always completely reliable and more than one source should be consulted.

Additionally, variations in the sequences used to design taxon-specific assays exists as for instance SNP can occur between the varieties of one plant species (Broothaerts et al., 2008; Papazova et al., 2010). The difficulty here is that information on the nucleotide sequence in different plant varieties is not available. This problem can be partially solved by designing the SYBR®Green primers on basis of existing TaqMan® taxon-specific assays for which experimental tests have been performed. Presence of SNP in the primer annealing sites can lead to a false negative result and to the conclusion that an event containing this target is not present when the assay is applied to an unknown sample (Broothaerts et al., 2008; Papazova et al., 2010).

Upon selection of the suitable sequence different primer pairs are designed by using appropriate bioinformatic tools. One of the most widely used programs is Primer3 (Rozen & Scaletzky, 2001). These primer pairs are further assessed *in silico* for their specificity. This can be done by means of bioinformatic tools such as the primer search module in the EMBOSS bioinformatic platform, BLAST searches etc. For transgenic elements, this theoretical specificity test is performed using sequences from authorised GM events. If the primers target a reference taxon-specific sequence, it should be tested if they are specific for the target taxon and do not amplify closely related species. Here, the criteria for specificity for reference assays of the event-specific quantification methods also apply (part 5).

As the goal is to use all the methods simultaneously under uniform conditions, particular attention is paid on the amplicon size and the primer annealing temperature (T_m) when developing the primers. Amplicons with a size lower than 100 bp are preferred although the size for real-time PCR amplicons can be as large as 250 bp. For qPCR detection smaller amplicons are favoured in order to avoid lack of amplification due to the possible fragmented status of the DNA in the sample (part 2). In addition, the melting temperature of the primers should be around 60°C according to the general requirements for qPCR primers (www.appliedbiosystems.com). The formation of primer dimers and hairpins should be checked and primer pairs showing this feature should be excluded for further analysis.

4.3 Validation of a SYBR®Green screening method

The in-house validation of a SYBR®Green screening method is based on the determination of several method characteristics that are required for the validation of event-specific quantitative methods (ENGL, 2008 - part 5), namely applicability, practicability, specificity, Limit of Detection (LOD), Limit of Quantification (LOQ) and precision (RSDr%). The definitions of these parameters can be found in the glossary. The GMOlab has developed its own experimental set up in order to assess these parameters. Upon validation the results are evaluated and if they meet the acceptance criteria the method can be used under accreditation. Additionally, the critical values which are introduced in the CoSYPS (part 6) are determined during the in-house validation.

The method is **applicable** when it detects the target in the respective GMO for which it was designed. To test this aspect of a method a list of GM events containing the target (positive samples) and events not containing the target (negative samples) is made. Usually, this list is limited to GM events which are authorised and for which (certified) reference materials are available. If possible, different matrices (e.g. gDNA, pDNA, raw material, processed material,…) are included and different GM concentrations are used. Further the applicability of the methods is assessed by screening certified reference materials which are used in the GMOlab for validation and calibration purposes.

The **practicability** of the SYBR®Green screening methods follows directly from the fact that all methods have been developed in-house. During the development, the use of the same conditions (qPCR program, reaction volume, …) and qPCR instruments have been taken into account. This will thus allow using all methods in a same run during routine analysis of a sample.

The **specificity** of the method is first assessed *in silico* (part 4.2) and further experimentally. The screening method should be specific for the target for which it is developed and should not be homologous and give an amplification product with other sequences. The specificity is experimentally tested on all materials to which the analysis can be applied. The GM events or taxa containing the target should give a positive amplification signal, while the ones which do not contain it should give no amplification signal. An amplification signal is considered positive when a C_t value and a melting curve analysis are recorded. Absence of amplification is considered when either no C_t is recorded or when a C_t value at least 10 C_t higher than the one of the positive samples is measured. To assess the nominal T_m value, a plasmid containing the construct under analysis may be used.

As the screening methods developed and validated at the GMOlab are based on the SYBR®Green detection chemistry, the melting temperature of the amplicon is an important parameter related to the specificity of the method. The melting temperature (T_m) of a DNA sequence is dependent on a large number of factors, among which the ionic conditions in the sample solution, the DNA nature (sequence, secondary structure, etc.) and the starting concentration of the DNA molecule (Hillen et al., 1981; Rouzina & Bloomfield, 2001). Moreover different qPCR instruments tend to measure slightly different values for a given amplicon (due to differences in heating block control, mathematical integration, extrapolation, etc.). The variation of the T_m follows a normal distribution and the T_m of the method is calculated as the average T_m from all the data obtained during validation. Additionally, a T_m confidence interval is calculated ($T_m \pm 3$ standard deviations) which is used further to decide whether the correct target has been amplified (part 6). The T_m and its

confidence interval can be updated regularly by adding data from analysis of routine samples to the existing dataset.

Using the data from the *in silico* and experimental specificity tests, mostly only one primer pair is selected for determination of the method sensitivity (LOD and LOQ) and repeatability.

To assess the **sensitivity** of the developed method, a GM event containing the target is used (usually a CRM with a known GM%). It should however be noted that the GM-specific CRM are certified for the content of a specific GM event and not for the content of the screening target (promoter, coding sequence, terminator). This demonstrates that the preliminary information on the elements inserted in a GM event and their copy number is crucial in order to estimate the correct copy number of the target. For taxon-specific markers, this assessment can be done using a wild type (non-GM) material. The LOD and the LOQ are determined on basis of serial dilutions starting from at least 2000 target copies until the theoretical zero copy numbers. Each of the dilutions is run in six replicates.

The **LOD** is set up at the level where less than 5% false negatives are observed (Codex Alimentarius, 2009). As it is not feasible to perform the analysis on a large number of PCR replicates, six repeats are run per dilution point. If all six repeats are positive, this means that 95% of the time a positive sample will indeed be detected. Therefore the LOD of the screening method is set at the haploid genome copy level at which all six replicates provide a specific positive signal (n = 6; 6/6 specific signals) (AFNOR XP V 03-020-2).

The **LOQ** is defined as the target copy number with a similar positive PCR result (expressed as C_t value) upon six-fold measurement of the target sequence in the same DNA sample with a minor standard deviation (SD_{Ct}<0,5) (AFNOR XP V 03-020-2). A screening target is in principle not quantified, but the LOQ can give an idea about the content of the target in an unknown sample.

Additionally, the **precision** (inter-run repeatability) of the method is determined. In practice this is done by calculating the relative repeatability standard deviation (RSDr%) on each of the dilutions used to determine the LOD and LOQ. Hereto, the experiment is performed under repeatability conditions (in a short period of time, on the same qPCR instrument by the same operator) in four independent runs. The RSDr% is calculated according to the ISO 5725-2. The method is accepted as valid when the RSDr% is below 25%.

4.4 Conclusion

As, to date, no instructions on the development and validation of screening methods are available, the laboratories need to set up their own experimental plan and criteria. At the WIV-ISP-GMOlab, development and validation of SYBR®Green methods for screening purposes is done in a harmonized way to allow applying the methods in a single qPCR run. The parameters evaluated, the way to perform this assessment and the acceptance criteria are based on previously published documents (ENGL, 2008; Codex Alimentarius, 2009; AFNOR XP V 03-020-2).

Upon evaluation of all the necessary parameters and their accordance with the set criteria, a validation dossier is established. The LOD, LOQ (expressed as a C_t value) and the T_m interval are introduced into the CoSYPS Decision Support System and serve as decision values to conclude if a sample is positive for the target or not (part 6). Subsequently the method is implemented in routine GMO detection under ISO 17025.

5. Validation of a qPCR method for GMO quantification and its implementation in a routine laboratory under ISO 17025 accreditation

5.1 Introduction into the legal context

Regulation (EC) 1829/2003 on genetically modified food and feed defines that food and feed products containing or derived from GMO must be labelled. The labelling requirements do not apply to food and feed containing GMO in a proportion not higher than 0,9% of the ingredients, provided that this presence is adventitious or technically unavoidable. Moreover, the recently adopted "Low Level Presence" Commission Regulation (EC/619/2011) requires a reliable quantification at a level of 0.1%. Member States are responsible for monitoring the GMO content of products and compliance with GMO labelling requirements. In this context, the enforcement of the EU legislation on GMO labelling requires GMO detection methods that are sound, precise and robust. It is, therefore, an essential requirement to use validated methods for GMO detection and quantification. Only in this manner it can be assured that independent control laboratories achieve comparable analysis results and are able to fulfil regulatory tasks (JRC, 2010).

The submission and validation of a GMO detection method is an integral part of the regulatory and approval process for GM food and feed to be placed on the market (EC/1829/2003). This Commission Regulation states that the application for authorisation should include, amongst others, "methods for detection, sampling and identification of the transformation event". As a consequence, the biotech companies have to provide detection protocols and control samples to validate the event-specific method to the EU-RL GMFF. These methods should be based on the real-time PCR technology (EC/787/2004). In view of the European harmonisation and standardisation of methods for sampling, detection, identification and quantification of GMO, the EU-RL has published a list of parameters to be tested and their acceptance criteria in the a document "Definition of minimum performance requirements for analytical methods of GMO testing" (ENGL, 2008).

A GM event cannot be authorised in the EU before a relevant detection method has been validated. The method validation process is conducted by the European Commission's Joint Research Centre (JRC) in its capacity as European Union Reference Laboratory for GM Food and Feed, and is assisted in its task by the European Network of GMO Laboratories. Commission Regulation EC/882/2004 establishes that analytical methods used for food and feed control must be verified by control laboratories before their use (JRC, 2010). In practice, after testing of the material and protocol, the JRC distributes the sample material and corresponding reagents to the participating laboratories in a ring trial. The validation ring trials are organised according to the requirements set up in ISO 5725 and following the IUPAC protocol (IUPAC, 1995). In such a collaborative validation trial, the EU-RL is assisted by the National Reference Laboratories (NRL) which are assigned as official control laboratories at national level (EC/882/2004). The NRL have to be accredited under ISO 17025 standard. Usually there are 12-13 participating laboratories, randomly selected from all available NRL. The validation ring trial aims at determining the method performance characteristics.

In this way the submitted method is evaluated with regard to the validation criteria. Failure to meet these criteria leads to rejection of the method and consequently to a delay in the authorisation of the GMO. Upon acceptance, the EU-RL GMFF prepares a validation report

with the results of the study and the validated protocol. These are submitted to the European Food Safety Authority (EFSA) and are subsequently published on the EU-RL GMFF official website. Upon publication the validated methods become official methods. The method validation thus provides the enforcement laboratories with standardised and harmonised methods applicable in official GMO detection.

5.2 Evaluated parameters for newly developed event-specific methods for GMO quantification

5.2.1 Evaluation of method performance characteristics by the EURL-GMFF

The requirements for method **specificity** are laid down in the legislation. The method submitted has to be event-specific (based on the specific sequence of the plant-transgenic construct junction, part 3) and should detect only the specific GMO submitted for authorisation to be useful for unequivocal detection/identification/quantification of the GM event (EC/641/2004). To demonstrate that the method is event-specific, it has to be tested against all GM events from the applicant which are currently authorised in different parts of the world and against those still in development.

As the submitted methods are quantitative, they also include a reference taxon-specific assay. The specificity of this assay should also be tested. For taxon-specific assays the target should be preferably a unique sequence present in a single copy in the target plant genome. The copy number and the specificity have to be assessed *in silico* by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches against known databases. In addition, the taxon-specific target should not show amplification signals with close relatives or taxa of the most important food crops. Usually, the different biotech companies develop their own taxon-specific method and test it on a range of taxa selected by them. This can pose several problems for the laboratories applying the methods. Firstly, there is no standard list of taxa and varieties to be included in the test. Ideally, the reference assay should be tested on a large range of varieties covering the existing natural variation within the taxon in order to assure that it will amplify any material from the plant species targeted by the method. Secondly, the existence of more than one reference system for events of the same plant taxon requires the use of several reference assays in quantification, which increases the costs of the analysis by the laboratory. In this context the requirements for the specificity of the taxon-specific reference assays should be made more precise and harmonisation in the methods used for different GM events is needed.

Information on the **applicability** of the method should be provided. This includes information on the scope of the method. In addition, information on known interferences with other analytes and the applicability to certain matrices should be supplied.

The **practicability** of the method should be demonstrated. For instance, methods where the reference and the event-specific assays are run on different PCR plates or under different PCR cycling conditions are less practicable and would be time and cost consuming when applied in a routine laboratory.

Besides these criteria, other parameters related to the method performance are assessed namely the **dynamic range, linearity, amplification efficiency, LOD and LOQ, trueness, precision and robustness**. The definitions of all parameters can be found in the glossary.

5.2.2 Evaluation of method performance characteristics, performed by the analysis of the results of the inter-laboratory collaborative trial

Once the EU-RL GMFF has made a scientific evaluation of the method based on the performance of the above-mentioned parameters (as provided by the method developer), it organizes a validation ring trial (concerning dynamic range, precision, relative reproducibility standard deviation and trueness). The participating laboratories receive the necessary samples and reagents and a detailed experimental protocol. It should be noted that the purpose of the ring trial is to assess the performance of the method and not of the laboratory. Therefore each participant has to follow the experimental procedure strictly. The results obtained by the laboratories are expressed as GM% for each tested level. These results are further scrutinised for outliers by the EU-RL GMFF using statistical methods recommended by ISO 5725. In addition, the mean value is calculated for each GM level analysed. Based on the parameters assessed during the ring trial, a conclusion is made on the compliance of the method with the ENGL method acceptance criteria and if it can be considered applicable in regard to the requirements of EC/641/2004.

5.3 Implementation of a validated event-specific method in a testing laboratory

When the interlaboratory validation study is completed and the method is considered as applicable, the method is ready to be implemented in routine testing laboratories like the GMOlab.

On the one hand, Commission Regulation EC/882/2004 states that official laboratories shall be accredited according to the ISO 17025 standard. An ISO 17025 accreditation, under a fixed or flexible scope, implies that "the laboratory shall confirm that it can properly operate standard methods before introducing the tests for calibrations". On the other hand, according to the same regulation, it is the task of the EU-RL GMFF to provide the NRL with details of analytical methods, including reference methods. In this context, guidelines for implementation of the validated methods in the routine laboratory are set up by the ENGL in the document "Verification of analytical methods for GMO testing when implementing interlaboratory validated methods" (ENGL, 2011). These guidelines reflect the requirements set up in the document "Definition of the Minimum Performance Requirements for analytical methods of GMO testing" (ENGL, 2008), but also give additional guidance on how to design the experimental set up and to calculate the required values. In practice the laboratories have to design the quantification experiment in which two or three GM levels are quantified and the parameters described hereunder have to be assessed.

Dynamic range, R^2 coefficient and amplification efficiency: these parameters can be calculated simultaneously from calibration curves when testing other parameters (trueness and precision). For each target, the average values of at least two calibration curves should be taken. The dynamic range should be tested between 1/10th of the threshold value and 5 times this value i.e. between 0,09% and 4,5% for the 0,9% labelling threshold. The PCR efficiency should be between 90 and 100% and the R^2 coefficient needs to be equal or above 0,98 to have a linear curve.

Trueness should be determined at a level close to the level set in the legislation (0,9%) or according to the intended use of the method and additionally at a level close to the LOQ. The trueness can be measured using a CRM or if not available on a sample from a proficiency test (PT). To comply with the acceptance criterion, the measured value should

not deviate more than 25% from the true value. In the case of a PT sample a z-score in the range of (-2;2) should have been obtained.

The **Relative Repeatability Standard Deviation (RSDr)** should be calculated on at least 16 single test results obtained under repeatability conditions. Repeatability should be available for all tested GM levels. The RSDr needs to be equal or below 25% to be acceptable.

Furthermore, the enforcement laboratory should estimate the **sensitivity** of the method. Hereto, four parameters can be calculated. The *Relative LOQ (LOQ$_{rel}$)* is estimated at low concentration(s) of positive material e.g. 0,1%. The LOQ$_{rel}$ is set at this level if the RSDr is below 25%. The *Absolute LOQ (LOQ$_{abs}$)* is estimated by measuring dilution series of low copy numbers of the target. The LOQ$_{abs}$ is set as the last dilution where the RSDr is lower than 25%.

The *Relative LOD (LOD$_{rel}$)* is estimated using ten replicates of a positive control material with a low GM level. The LOD$_{rel}$ is set at this level if the ten replicates show a positive amplification. The *Absolute LOD (LOD$_{abs}$)* is estimated as the copy number at which not more than 5% false negatives are obtained. In practice this is performed by evaluating ten PCR replicates of low copy number of the target. The LOD$_{abs}$ is set at this level if the ten replicates score positive.

5.4 Conclusion

A GMO quantification method filed by the biotech companies together with the application for authorisation follows different steps. Firstly, the developer needs to provide information on the performance of the method. Hereto, he needs to evaluate different parameters as laid down in the ENGL document (ENGL, 2008). Secondly, the EU-RL GMFF evaluates the submitted information and decides whether the dossier is in compliance with the set criteria. Thirdly, the EU-RL organises a ring trial to validate the method. Hereto it gets the support of the different NRL that participate in the validation. Fourthly, the enforcement laboratories need to assess a number of parameters before to implement the method in their laboratory for routine analysis under ISO 17025 accreditation.

At WIV-ISP-GMOlab, the assessed parameters and the data obtained during the in-house verification are gathered in a validation dossier. The event-specific method is in a first time used as a qualitative identification method in the second step of GMO analysis. The critical parameters determined during the in-house validation for these methods are the LOD$_{abs}$ and LOQ$_{abs}$. These parameters, expressed as C$_t$ values, are introduced into the DSS and serve as a threshold to decide if the GM event is present in the sample and in case of presence if it is quantifiable.

For quantification methods, no real DSS exists but different parameters are evaluated at each use in routine analysis and have to be in compliance with the set criteria. In a first step, the parameters of the calibration curves of the event-specific and the taxon-specific method (linearity, slope, PCR efficiency) are evaluated. Additionally, control samples (0,1% and 1%) are quantified and the result has to fulfil the acceptance criterion for trueness. In this way the obtained quantitative results for unknown samples are validated.

6. Introduction of the qPCR methods in the Decision Support System (DSS)

6.1 General strategy

As described before (part 3), to cover the broadest GMO spectra, SYBR®Green qPCR methods have been developed and validated in the GMO detection platform. In this context,

it rapidly becomes tedious in routine analyses to manually combine all the screening results in order to decide which GMO are potentially present in a sample. Therefore, in support to the qPCR data, a simple mathematical model has been developed to automatically calculate the possible presences in a product based on the outcome of the qPCR screening analysis (Van den Bulcke et al., 2010). The CoSYPS, standing for Combinatory SYBR®Green qPCR screening, represents a novel tool for GMO analysis based on the SYBR®Green qPCR technology. Using this decision support system alone is not sufficient. The suspected GM events need to be specifically identified in a second step, using e.g. the EU-RL Taqman® event-specific qPCR method(s). In a third step, the positively identified GM events are quantified to asses if their content complies or not with the 0,9% labelling threshold (EC1830/2003).

This newly developed tool is a versatile, cost-effective and time-efficient approach in assessing the GMO presence in analytical samples and can be applied in routine analysis for enforcement purposes. The full system has been patent protected (Van den Bulcke et al., 2008).

Here the construction, functioning and the theoretical basis of the CoSYPS will be described. Further explanation on the mathematical functioning of the CoSYPS may be found in the recently published paper "A theoretical introduction to "Combinatory SYBR®Green qPCR screening", a matrix-based approach for the detection of materials derived from genetically modified plants" (Van den Bulcke et al., 2010).

6.2 Screening for GMO candidates by CoSYPS analysis

The CoSYPS is based on the determination of the presence of certain element(s) originating from GMO and plant taxa frequently occurring in food and feed products. Hereto, SYBR®Green qPCR analysis of gDNA extracted from the product is performed, using primer pairs targeting different (multiple) discriminatory marker amplicons (part 3 and table 2).

During the SYBR®Green qPCR analysis of the sample, two critical qPCR parameters are recorded for each method used: the C_t and T_m values. Within the Decision Support System the obtained values are then compared to the LOD (expressed as a C_t value – see glossary) determined in the validation of the qPCR screening method and the nominal T_m value of the amplicon (see glossary). Both parameters are used as decision criteria for the analysis and are incorporated as such in the CoSYPS Decision Support System.

In a first step, the CoSYPS algorithm compares the measured C_t and the T_m values for each screening element with the corresponding "decision values" in the DSS. The latter values are determined during the in-house validation of the method (part 4). A signal generated in SYBR®Green qPCR analysis for a sample is considered as positive by the CoSYPS when an exponential amplification below the C_t value of the LOD (+ 1 C_t) is obtained and the amplicon has a T_m value that falls within the determined T_m confidence interval (part 4). In agreement with the decision principles of the ISO norm 24276 (twice positive, twice negative), all decisions within the CoSYPS are based on the extraction and analysis of two distinct representative sub-extracts and eventually confirmed by a third analysis in case of ambiguous results (one positive, one negative). Therefore, a sample is positive for a specific screening element when the C_t and T_m results are unambiguously for both sub-extracts. Any positive signal obtained with a SYBR®Green qPCR method targeting a particular GM element indicates that a GMO comprising this target could be present in the sample. When several GMO contain the same target, a positive result generated by this screening method indicates that potentially all these GMO may be present in the sample. However, when

multiple targets are present in a GMO and the CoSYPS contains methods for each of these targets, all targets present in that GMO must be positive to conclude that this GMO might be present.

The second step in the CoSYPS algorithm is based on a mathematical model. A unique prime number (a prime number is a natural number that has exactly two distinct natural number divisors: 1 and itself) is associated with each particular screening method. When the sample is considered positive for a certain screening element, this specific prime number is assigned to the sample. When it is considered negative, the number 1 (neutral element in multiplication) is assigned. By multiplying all assigned values, the algorithm calculates the "Gödel prime product" (GPP_{sample}) of the sample (the product of the prime numbers corresponding to the positive scoring screening methods). In a similar way each GMO can be represented by a product of the different prime numbers corresponding to the elements belonging to the GMO. This product is designed as the "Gödel prime product" (GPP_{GMO}) of the GMO and represents a "mathematical tag" for this GMO. Note that several GMO can be associated with a same GPP product as they comprise the same genetic elements.

The third step of the CoSYPS is based on the fact that, as a consequence of the nature of prime numbers, the division of the GPP by any of the prime numbers used in the generation of the GPP is an integer. Therefore the presence of a target in a GMO can be mathematically traced by generating this fraction: the program makes the ratio between the GPP_{sample} and the GPP_{GMO} to identify which GMO could be present in the sample (the division generates an integer).

Consequently, on the basis of the positive signal(s) obtained during the screening for each specific SYBR®Green qPCR method, the specific prime number assigned to each method is scored by the CoSYPS. The multiplication of these prime numbers allows the CoSYPS to calculate the GPP for the analysed sample. From this number, the CoSYPS can select all the potential GMO present in the sample by a series of simple divisions.

6.3 Integration of an event-specific method in the Decision Support System and interpretation

On the basis of outcome of the CoSYPS analysis a set of candidate GMO which could possibly reside within the product can be identified. In order to confirm the presence of a certain GM event in this product, event-specific Taqman® qPCR analysis is performed in a next step by applying methods validated and published by the EU-RL (http://gmo-crl.jrc.ec.europa.eu).

During the sample analysis, the C_t value obtained as outcome of the event-specific qPCR is recorded. This C_t value is compared to the LOD and LOQ (as determined during the verification of the identification method in the laboratory - part 5). These values were previously introduced in the Decision Support System.

A GM event is considered detectable by the DSS when an exponential amplification below the C_t value of the LOD (+ 1 C_t) is obtained. The LOD was obtained under repeatability conditions (part 4).

To conclude which GM events are effectively present and identified in the sample, the DSS retains all prime numbers of the GM event with a C_t value below the C_t value of the LOD (+

1 C_t) threshold level. The C_t value is also compared with the LOQ + 1 C_t to decide if the GM event is present at a quantifiable level.

If no authorised GMO can explain the presence of a set of screening targets, it can be concluded that the sample contains one or more unassigned targets. The unassigned signals are mostly due to unauthorised GMO or donor organisms (bacterial, viral and plant sources of transgenic elements). In such cases more complex analysis like DNA walking, DNA sequencing has to be performed outside of the routine to elucidate their origin.

6.4 Practical case

As an example, the accredited SYBR®Green qPCR methods available in a qPCR platform for GMO detection and their associated prime numbers are p35S, tNOS, pNOS, t35S, CryIAb, PAT/pat, CP4, PAT/bar for the transgenic elements and ADH1, LEC and CRU for the taxon-specific markers (table 3a). The elements targeted by these methods can be found in part 3 table 2.

During the screening analysis a positive signal (correct T_m and Ct < C_t of LOD + 1 C_t) is found for the p35S, tNOS, CryIAb, t35S , PAT/pat and ADH1 elements while no positive signal was obtained for pNOS, CP4, PAT/bar and the other species-specific targets (table 3b–step1). For each positive screening marker (p35S, tNOS, CryIAb, t35S and PAT/pat and ADH1) the specific prime number is assigned to each of the corresponding methods. As the pNOS, CP4, PAT/bar targets and the other taxon-specific markers are considered as negative the assigned number for all of these methods is 1. The "Gödel prime product" of the sample (= 1057485) is calculated by multiplying all the assigned prime numbers (table 3b-step2). The CoSYPS will compare this GPP$_{sample}$ with the GPP of all GM events that have previously been introduced in the system. The example is given here for four GM events.

The transgenic MON 810 and T25 events are described as a function of three transgenic elements (p35S, tNOS, CryIAb) and (p35S, t35S, PAT/pat) respectively and one maize-specific (ADH1). The GA21 maize is covered by the tNOS and maize–specific element. The GTS40-3-2 event is defined by three transgenic elements (p35S, tNOS, CP4) and the soybean endogen (LEC). Consequently, the "Gödel prime product" of the MON 810, T25, GA21 and GTS40-3-2 are 5655 (= 3 X 5 X 13 X 29), 16269 (= 3 X 11 X17 X 29), 145 (= 5 X 29) and 8835 (= 3 X 5 X 19 X 31) respectively (table 3b-step 3).

To assess which GMO are potentially present in the sample, the "Gödel prime product" of the sample is divided by the GPP of each GMO (table 3b-step 3). The result is an integer only for MON 810, T25 and GA21. From the screening analysis, the CoSYPS thus predicts that MON 810, T25 and GA21 are potentially present while GTS40-3-2 is not. As a consequence MON 810, GA21 and T25 have to be further analysed with the event-specific method to confirm their presence.

In order to confirm the presence of MON 810, T25 and GA21 in the sample product, the event-specific qPCR analyses are performed. The results (expressed as C_t values) confirm the presence of MON 810 and T25 while GA21 is not detectable (table 3c). The C_t values obtained are compared with the LOQ + 1 C_t of each method and show that only MON 810 can be quantified. Finally the GM% of this event will be compared to the labelling threshold (0,9% mass per ingredient) in order to conclude on the conformity of the sample.

a. Accredited SYBR®Green qPCR available

Screening methods	p35S	pNOS	t35S	tNOS	CryIAb	PAT/pat	PAT/bar	CP4	ADH1	LEC	CRU
Prime numbers	3	7	11	5	13	17	23	19	29	31	37

b. CoSYPS algorithm and screening methods

CoSYPs step1 - Sample analysis "+" is assigned when value < LOD+1 C_t; "–" is assigned when value > LOD + 1 C_t											
Subsample 1	+	-	+	+	+	+	-	-	+	-	-
Subsample 2	+	-	+	+	+	+	-	-	+	-	-
CoSYPS step 2 - Calculation of the Gödel prime product of the sample											
Product of	3	1	11	5	13	17	1	1	29	1	1
GPP		1057485									

CoSYPS step 3 - Assessment of potential GMO present in the sample			
GMO	GPP of GMO	GPP$_{sample}$/ GPP$_{GMO}$	Decision
MON810	5655	187	Confirmation by event-specific Taqman method
T25	16269	65	Confirmation by event-specific Taqman method
GA21	145	7293	Confirmation by event-specific Taqman method
GTS 40-3-2	8835	119,69	No confirmation by event-specific Taqman method

c. DSS and confirmation by event-specific Taqman method

Taqman				
	MON 810	T25	GTS 40-3-2	GA21
Subsample 1	LOD > C_t < LOQ	LOD > C_t > LOQ	■	LOD < C_t > LOQ
Subsample 2	LOD > C_t < LOQ	LOD > C_t > LOQ	■	LOD < C_t > LOQ
Results	*Present Quantifiable*	*Present*	■	*Not detectable*

Table 3. Mathematical functioning of the CoSYPS, allowing demonstrating the possible presence of a set of GMO in a product based on the outcome of a qPCR screening analysis.

6.5 Conclusion

By combining the results of the screening analysis, the CoSYPS allows to decide in a fast way which GM events are possibly present in the sample under analysis. The use of the mathematical algorithm, which compares the GPP$_{sample}$ and GPP$_{GMO}$, excludes the need for manual calculations and comparisons. The only thing that needs to be done by the operator is the preliminary introduction of the critical values (C_t corresponding to the LOD and LOQ, T_m) obtained during method validation in the system. Further, in identification, the obtained results for a sample are compared with the LOD and LOQ values determined during in-house validation of the event-specific methods. From this comparison, the Decision Support System will indicate which GM events are present and at which level and thus allow deciding which GMO needs to be quantified in the sample. This Decision Support System, developed and patented by the WIV-ISP-GMOlab is thus a very efficient, user friendly and cost-saving tool in GMO detection.

7. Conclusion

In the near future, the number and the diversity of GM crops will continue to increase, as well as the requests for authorisation for their import for food and feed in the EU. Beside the notifications of GM events produced by multinational biotech companies, many GM events will be developed by universities, national research centres and small private companies. Thus, the chance for accidental occurrence of unapproved GMO in the EU food and feed chain trough importation will be higher. As the EU's general policy supports strong commitment to consumer protection and freedom of choice, and therefore mandatory product labelling, the development of sensitive, reliable but also cost-effective and flexible strategies for the detection of GMO in products through establishment of molecular platforms will become more and more crucial.

The GMO detection platform developed at WIV-ISP consists of a pre-PCR step namely DNA extraction and three consecutive qPCR phases. In this view, the choice of efficient methods to extract good quality DNA, in particular for processed food and feed, is a critical factor. A pre-PCR evaluation of the extracted gDNA is necessary as well as setting criteria for the purity and integrity of the DNA. Furthermore, the presence of PCR inhibitors is a major obstacle for efficient amplification in qPCR. This step may even become more important as the number of GM plant taxa becomes larger. Developing simple standard methods for genomic DNA extraction minimizing inhibition will therefore be the key for providing concordant results when using qPCR techniques.

Due to the broad range of GMO that my occur in the EU food and feed chains, the use of screening strategies only based on the 35S promoter of the Cauliflower Mosaic Virus (p35S) and the nopaline synthase terminator of *Agrobacterium tumefaciens* (tNOS) followed by the analysis of the sample with event-specific EU validated methods by the enforcement laboratories will become insufficient. As a consequence, new methods focusing on an intensive screening analysis need to be developed.

At the present time several high-tech strategies like multiplex PCR and consecutive detection and identification of the amplification products using micro-arrays (Chaouachi et al., 2008, Morisset et al., 2008, Hamels et al., 2009) or PCR combined with capillary electrophoresis (Nadal et al., 2009) have been proposed to deal with this discriminative problem and the broad diversity of GMO. However, at the present time, these technologies require additional costly equipment and investments in technical support. Furthermore, they need technological optimalisation as they show a high background at low target level. These difficulties make them less suitable for routine or enforcement purposes.

Contrary to the above-mentioned technically complex strategies, our approach based on numerous singleplex qPCR-based methods developed to function under the same reaction conditions combined with the informatics decision support tool CoSYPS may in the future represent a very effective alternative. This newly developed tool is considered as a versatile, cost-effective and time-efficient platform assessing the GMO presence in analytical samples. In addition, it functions in routine analysis for enforcement purposes in a commonly applied 96-well plate qPCR format.

In the future, the research of the molecular platform of the WIV-ISP will focus on the development of more discriminative SYBR®Green qPCR screening methods to cover the

broad range of GMO and UGM and thus to improve the resolution of the system. Particular importance will be given to their use in a modular approach associated with a decision tree cascade. Moreover, our strategy aiming at developing harmonised SYBR®Green qPCR screening methods incorporated in the Combinatory SYBR®Green qPCR Screening (CoSYPS) system has a potential to be applied in other scientific fields than GMO detection. The application of this strategy for food borne pathogenic bacteria is now under development in our team.

8. Glossary

Amplification Efficiency

The amplification efficiency is the rate of amplification that leads to a theoretical slope of – 3,32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation:

$$Efficiency = 10^{\left(\frac{-1}{slope}\right)} - 1 \tag{1}$$

Applicability

Applicability is the description of analytes, matrices and concentrations to which the method can be applied.

Certified Reference Material (CRM)

A Certified Reference Material is a reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

Correlation Coefficient (R²)

The R^2 coefficient is the correlation coefficient of a (calibration) curve obtained by linear regression analysis.

Dynamic Range

The dynamic range is the range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision.

European Food Safety Authority (EFSA)

EFSA is an agency of the EU that provides independent scientific advice and communication on all matters concerning food and feed safety.

European Network of GMO Laboratories (ENGL)

The European Network of GMO Laboratories is a platform of EU experts that plays an eminent role in the development, harmonisation and standardisation of means and methods for sampling, detection, identification and quantification of Genetically Modified Organisms (GMO) or derived products in a wide variety of matrices, covering seeds, grains, food, feed and environmental samples. The network was inaugurated in Brussels on December

4th 2002 and it currently consists of more than 100 national enforcement laboratories, representing all 27 EU Member States plus Norway and Switzerland. Its plenary meetings are open to particular observers, such as to representatives from Acceding and Candidate Countries.

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

The core task of the EU-RL GMFF is the scientific assessment and validation of detection methods for GM Food and Feed as part of the EU authorisation procedure. The Joint Research Centre (JRC) of the European Commission and, more precisely, the Molecular Biology and Genomics Unit of the Institute for Health and Consumer Protection (IHCP), has been given the mandate for the operation of the EU-RL GMFF. Activities are carried out in close collaboration with European Network of GMO Laboratories (ENGL).

Genetically Modified (GM) event

A GM event refers to the unique DNA recombination event that took place in one plant cell, which was then used to generate entire transgenic plants

Genetically Modified Organism (GMO)

A Genetically Modified Organism is officially defined in the EU legislation as "organisms, not from human origin, in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination"

Limit of Detection (LOD)

The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected but not necessarily quantified, as demonstrated by single-laboratory validation.

Limit of Quantification (LOQ)

The limit of quantification is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Melting temperature (T_m)

The melting temperature is the temperature at which 50% of the DNA is single stranded.

National Reference Laboratory (NRL)

A National Reference Laboratory on GMO operates in the frame of Commission Regulation EC/1829/2003 on GM Food and Feed and Commission regulation EC/1830/2003 on labelling and traceability of GMO. It assists the EU-RL and the NRL from the different member states are gathered in the ENGL.

Practicability

Practicability is the ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. cost/sample) of the method.

Precision - Relative Repeatability Standard Deviation (RSD$_r$%)

The relative repeatability standard deviation is the relative standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test

results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Precision – Relative Reproducibility Standard Deviation (RSD$_R$%)

The relative reproducibility standard deviation is the relative standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where the test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Reference material (RM)

A Reference Material is a material that is sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process.

Robustness

The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Specificity

Specificity is a property of a method to respond exclusively to the characteristic or analyte of interest.

Threshold cycle (C$_t$)

The threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C$_t$ value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons has been accumulated.

Trueness

The trueness is defined as the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

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10. References

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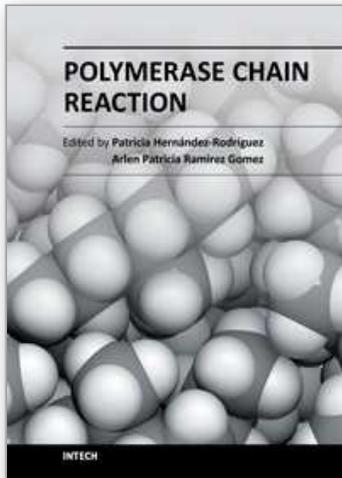
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