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GMOseek Final Report (Part 1)

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GMOseek appendices are contained in parts 2-5 of the report (see pages 33-34 for details).

Start date of project: 01 June 2009

Duration: 24 months

Project coordinator name: Dany Morisset

Project coordinator organisation name: National Institute of Biology, Slovenia

Executive summary

In recent years, an increasing number of countries have adopted labelling policies for genetically modified (GM) food to deal with issues such as food safety, and consumers' right of choice. In the European Union (EU), the GM Food and Feed Regulation (EC) 1829/2003 lays down labelling requirements for genetically modified organisms (GMOs) and products containing GM material. This stipulates that for any food or feed product containing a GM ingredient, this must be declared on the label.

In most EU enforcement laboratories, the standard testing technology is real-time polymerase chain reaction (qPCR). Different genetic elements can be targeted by qPCR. On the one hand, genetic "screening" elements that are found in several different GMOs can be used to detect the presence of GMOs, and on the other hand, "event-specific" targets are used for the identification of individual GMOs. The currently used GMO testing strategies consist of two phases: a screening phase followed by an identification phase. In the screening phase, GMO presence is detected by performing a minimal number of screening tests that cover most (or all) GMOs in question. If GMO presence is found, a second phase using event-specific tests is performed to identify the GMOs in the sample. EU enforcement laboratories face difficult challenges in the detection of GMOs. This is partly due to the ever increasing number, variety and complexity of authorised GM events, but also to the increasing problem of unauthorised GMOs (UGMs), for which EU validated methods of detection do not or rarely exist. EU enforcement laboratories must therefore detect an increasing range of GM events on the one hand, and manage to do so in a time- and cost-efficiency manner on the other hand.

The GMOseek project has primarily aimed at providing improved screening methods and strategies for a more cost and time-efficient analysis and detection of GMOs including unauthorised events which are the most relevant for the food safety issue. To do so, the project focused on the development of 1) new bioinformatics tools to design more efficient screening strategies; 2) new screening methods to be introduced in enforcement laboratories for a more pragmatic detection of authorised and also unauthorised GMOs in parallel.

First, theoretical and experimental knowledge regarding the genetic elements introduced in GMOs were compiled and thoroughly verified in a simple comprehensive Excel table named "GMOmatrix". Part of this information was also experimentally verified within the project, when practical. Then a user-friendly bioinformatics system named "GMOseek algorithm" was created to exploit the information contained in this GMOmatrix. The GMOseek algorithm selects from the GMOmatrix an optimal set of genetic elements that need to be targeted for screening purposes, suggesting the development of new screening methods if necessary. In addition, the bioinformatics system also helps the analyst in the enforcement laboratory throughout the whole experimental GMO analysis by analysing the experimental results, suggesting which GMOs are absent and which ones may be present in the sample, verifying the results

and alerting the analyst when inconsistent results, potentially due to the presence of unknown unauthorized GMOs (UGMs), are observed. When tested on real routine samples, the GMOseek algorithm proved to offer significant profit in terms of analysis cost, and to ensure wider coverage of GMOs in the sample. A simple Excel-based program to sort important screening methods was additionally developed for a more limited use. The GMOseek algorithm and the GMOmatrix will be beneficial to all enforcement laboratories, inside and outside the EU, wanting to improve their screening strategies, and will be made fully available upon the completion of the GMOseek project.

The second main objective of this project was to develop new qPCR screening methods targeting genetic elements incorporated into old and/or next-generation GMOs. A total of nine qPCR methods targeting unique genetic elements (singleplex qPCRs) and three qPCR methods targeting several genetic elements in a single reaction (multiplex qPCRs) where developed. The advantage of multiplex qPCR methods is that they significantly decrease the overall number of qPCR reactions needed, and thus also the time and cost of analysis. All the methods were developed in order to be easily applied in all enforcement laboratories, without further investment in material or reagents. Furthermore, used in combination with other screening methods, these methods add significant information to the screening phase results, helping to discriminate which GMO event(s) may be present in a sample. The multiplex methods developed in this project allow an almost full coverage of all authorized GMOs in the EU, as well as numerous non-authorized ones. From the twelve qPCR methods developed in the project, nine were in-house validated at the developer's laboratory and verified in a second laboratory, proving they are robust and practical. These methods showed high sensitivity to detect very low traces of targets in the sample, and great specificity to the target.

To support the further validation of the qPCR methods, the partners of the GMOseek project have produced guidelines describing the parameters one should measure to verify the performance of a screening method. Practical procedures to measure these parameters were given as well as acceptance criteria to decide whether these parameters are satisfying or not for GMO detection. These guidelines will be followed to complete the in-house verification of the methods as well as for the inter-laboratory trials. Moreover, these guidelines have also been distributed to the European Network of GMO laboratories (formed by almost 100 national enforcement laboratories) via the working group on Method Performance Requirements where it is expected that they could be used to update already existing official guidelines that are currently being re-evaluated. It is also expected that these guidelines will be beneficial to laboratories in other domains than GMO detection, where PCR detection methods are needed (e.g. food microbiology, environmental testing...).

In addition to the classical qPCR technique, an alternative DNA-based amplification technology named NASBA Implemented Microarray Analysis (NAIMA) was assessed as a technology able to offer further multiplexing for GMO detection when combined with microarray hybridization. Although the NAIMA method proved to be very promising for higher multiplexing, the microarray-based approach failed to

provide reliable detection; some GMOs present in samples remaining undetected. Therefore, this approach was considered as not practical and not adequate for GMO detection unless significant improvement is made.

Regarding the results of the bio-informatics activities, it is recommended to set-up a website in which the GMOmatrix and the GMOseek algorithm, optimised for web interface, would be freely and constantly available to all potential users. The starting Dutch-German EUGINIUS project will generate tables similar to the GMOmatrix; this matrix adapted to the GMOseek algorithm should ensure longer term availability of the bio-informatics part of the GMOseek project. This matrix approach and its associated bio-informatics tools could also be adapted to other PCR-based analyses where screening methods are needed.

Finally, nine novel qPCR assays developed in this project have been in-house validated and one assay is close to in-house validation completion. Before these qPCR methods can be applied for routine GMO detection in enforcement laboratories, it is recommended that further experimental verification is performed to add confidence to the observed satisfactory results. Then, the methods may be tested in a small-scale group before entering a large-scale validation, or"inter-laboratory trial", that would provide evidence of their fitness-for-purpose for GMO detection. The validated methods will then be made available to all enforcement laboratories, worldwide, to improve the experimental screening phase of GMO testing.

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List of abbreviations

BCCM: Belgian Co-ordinated Collections of Micro-organisms

CoSYPS: Combinatory SYBR®Green PCR Screening

CRL-GMFF: community reference laboratory for genetically modified food and feed

CRM: certified reference material

Ct: threshold cycle (in PCR)

ENGL: European network of GMO laboratories

EU: European union

gDNA: genomic DNA

GM: genetically modified

GMO: genetically modified organism

JRC: Joint research centre

LOD: limit of detection

NAIMA: NASBA implemented microarray analysis

NASBA: nucleic acid based sequence amplification

PCR: polymerase chain reaction

qPCR: real-time polymerase chain reaction

SOP: standard operating procedure

UGM: unauthorised GMO

WP: work package

WT: wild type

Project results

The GMOseek project has been organised in four work packages (Figure 1). WP1 activities were focused on the development of a GMOmatrix and two bioinformatics tools to make use of this GMOmatrix. WP2 activities included the development, in-house validation and transfer to second laboratory of novel singleplex screening Taqman® real-time PCR methods, novel screening SYBR®Green real-time PCR methods, multiplex screening Taqman® real-time PCR methods and the experimental evaluation of screening elements to verify the GMOmatrix. WP3 was dedicated to the development for NAIMA, an alternative to PCR-based amplification with microarray hybridization technology for detection of the targets. The WP4 was focussed on two aspects: the production of reference materials necessary for the development, in-house validation and transfer of screening methods from the GMOseek project. The second aspect was the production of validation guidelines to define the parameters needed to be checked during in-house and full validation of screening (qualitative) methods, the procedure to verify these parameters and their associated acceptance criteria. The guidelines were planned to be used for further validation of the methods developed during the GMOseek project.

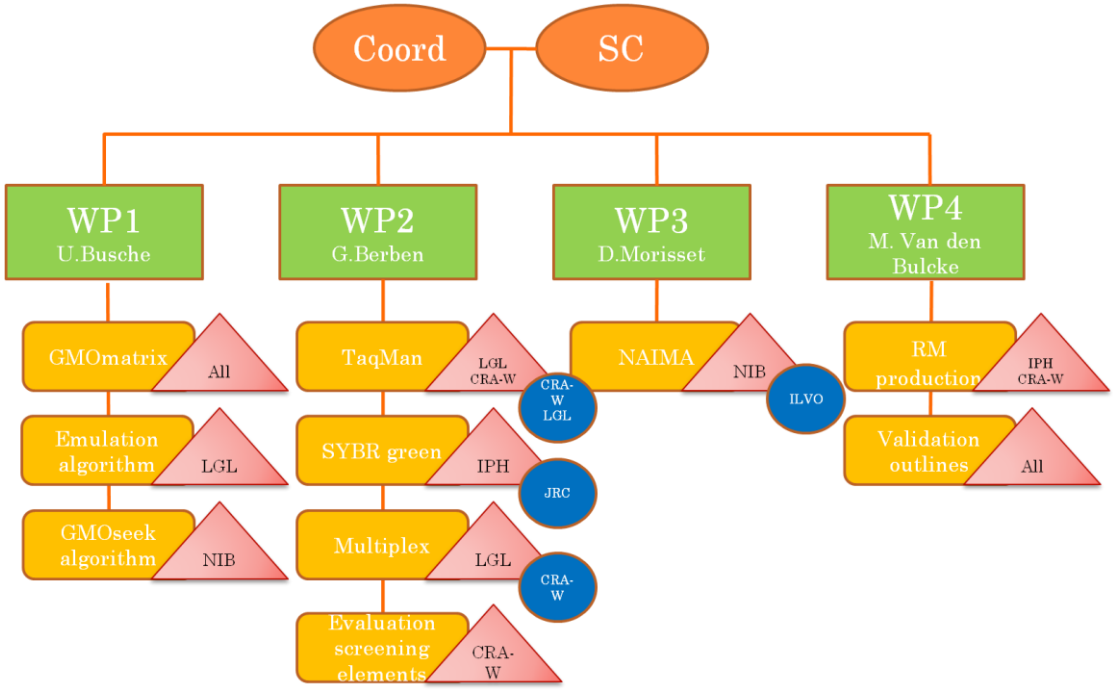

Figure 1: Organisation of the GMOseek project

The project activities (orange rounded rectangles) were divided in four work packages (green rectangles, name of the WP leader is given). The name of the responsible partner for each task is given in red triangles. After development and in-house validation in WP 2 & 3, the screening methods were transferred to a second laboratory indicated by blue circles. Finally, the whole project was coordinated by Dany Morisset (NIB) with the help from the steering committee (SC)

WP1: Bioinformatics

One objective of the WP1 was to be able to anticipate an upcoming situation for the next years where various 'asynchronous authorisations' for GM events in different EU member states and other countries may enter the European market. For this, recent works performed in individual laboratories and within diverse ENGL (European Network of GMO laboratories) working groups suggested that the so-called "matrix-based" approach would be beneficial for better GMO detection. In its "Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials", the ENGL ad hoc working group on "unauthorised GMOs" redefines the notion of matrix-based approach for GMO detection (document in final review before official publication in 2011 as JRC Scientific and Technical report from the JRC, by the Publications Office of the European Union). A relation matrix ("GMO matrix") must be established. This matrix shows the expected response by individual GMO events to specific PCR tests. This matrix is the basis for the term "matrix approach". In this approach, a series of PCR tests is first performed. The combined outcome of these tests is then systematically compared to the response to the performed tests by each of the GMOs, predicted in the matrix. Based on these comparisons, it is possible to conclude on: 1) which GMOs that are not detected (for these GMOs one or more elements/analytes is not detected; 2) which GMO that can be present (all elements are detectable), and 3) which additional PCR test(s) that could be used for further discrimination among the putatively remaining GMOs (ENGL ad hoc working group on "unauthorised GMOs" 2011).

Task 1: In-house GMO database provision, check-up and maintenance

[Relevant appendix: D1/01 - Model GMO matrix – Report]

A GMO matrix (called "GMOmatrix") has been built up in order to provide information regarding all the genetic elements constituting GM events. These genetic elements can be used as targets for new screening methods for detection of GM plant ingredients in food, feed and seed. All data forming this GMOmatrix were provided by the project partners and official databases (Task 1.1) and was thoroughly checked and corrected by laboratory analysis (in silico and experimentally) (Task 1.2). This Excel based matrix was continuously elaborated to facilitate a convenient selection of possible candidate genetic elements for detection and method development purpose. This GMOmatrix was the object of the deliverable D1/01 - Model GMO matrix. Report (see appendix D1/01). The GMOmatrix, regularly updated (Figure 2) (Task 1.3) was used as a basis for deciding which screening methods should be effectively developed within this project.

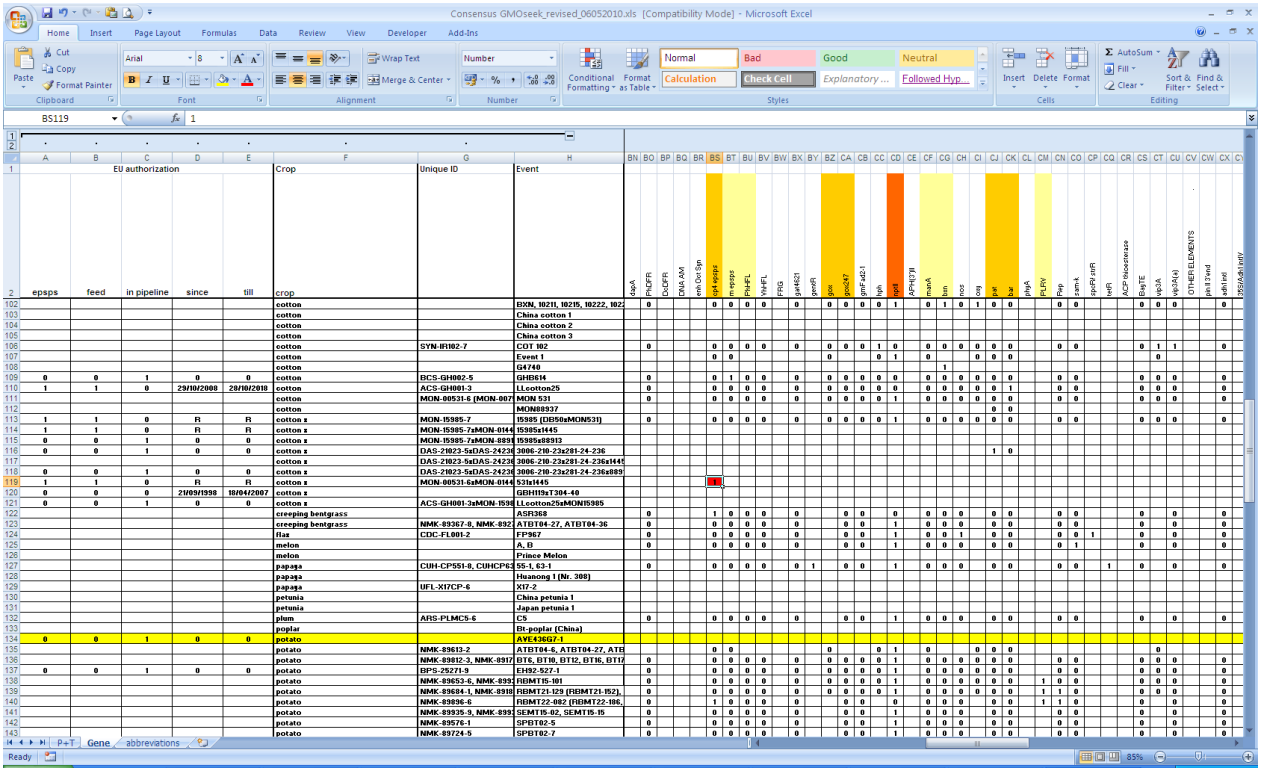

Figure 2: Screenshot of the GMOmatrix after check-up and update (May 2010)

Another goal of the GMOmatrix was its integration in two bioinformatics tools: the emulation algorithm of the EUGINIUS database (Task 2) and the GMOseek algorithm (Task 3).

Task 2: Development of a software to automatically generate GMO matrices

[No relevant appendix]

EUGINIUS will be a web-based database with many modules providing information such as the currently existing GM events (commercialized, in pipeline for commercialization, ,at development stage...), genetic elements composing these events, screening methods developed so far, authorization status, etc... The prototype EUGINIUS database has been already partially fed by the GMOmatrix developed in the GMOseek project. Furthermore, one of the modules associated to this EUGINIUS will be an export tool to automatically retrieve the necessary information under a format suitable for use with the GMOseek algorithm. The programming of the emulation algorithm (Task 2) depended on the programming of the EUGINIUS-MOREG database. Due to a delay of the European call for the programming of the EUGINIUS-MOREG database, Task 2 could not be started before February 2011, when the final choice for the subcontractor of BVL was taken. The subcontractor of the BVL and LGL was decided to be Opitz

Consulting, Germany. The programming of the EUGINIUS-MOREG therefore started towards the end of the GMOseek project (Spring 2011). LGL has already contacted Opitz Consulting for the development of an export module within the EUGINIUS database that will allow the production of tables compatible with the GMOseek algorithm. The planning of the procedure is in progress and the export module should be available at the end of 2011. The EUGINIUS database was originally fed with the GMOmatrix from GMOseek (as well as other sources of information about GMOs). As it will be constantly updates and thanks to its export module, it will allow the generation of updated GMOmatrix, compatible with the GMOseek algorithm, even after the GMOseek project ends.

Task 3: Selection of new potential genetic elements to be targeted

[Relevant appendix: D3/01 - GMOseek algorithm analysis performed - Report]

The GMOseek algorithm was developed for searching optimal combinations of screening methods, and therefore for selecting new potential genetic element targets in the screening phase of GMO detection.

Several computations were performed simulating situations for a real laboratory performing routine GMO detection (for instance the GMO laboratory in NIB). These computations demonstrated that the algorithm provides combinations of genetic elements that should be targeted by screening methods for more cost-efficient detection of GMOs (Figure 3) and better coverage of possible GMOs present in a sample (Figure 4).

Also changes in GMO frequency of appearance in foodstuff were simulated, proving the robustness of the algorithm to the changes that will occur with GMOs in food in the near future. Within the numerous results obtained during these simulations, several genetic elements appear often in the proposed combinations from the GMOseek algorithm. They can therefore be considered as important for improving GMO screening and should be targeted in new methods to be developed. These results were gathered in the deliverable D3/01 (see appendix).

The software tool was developed with an inspection panel consisting of a simple and user-friendly DSS guiding the analyst through the screening and the identification phases to the final qualitative analytical results, warning in case the screening results are contradictory with identification results. Such contradiction should be carefully checked as it may result from the presence of non-authorized GM events. Also, a warning signal is produced if several GM events are present in the sample as this may result from stacked GM events.

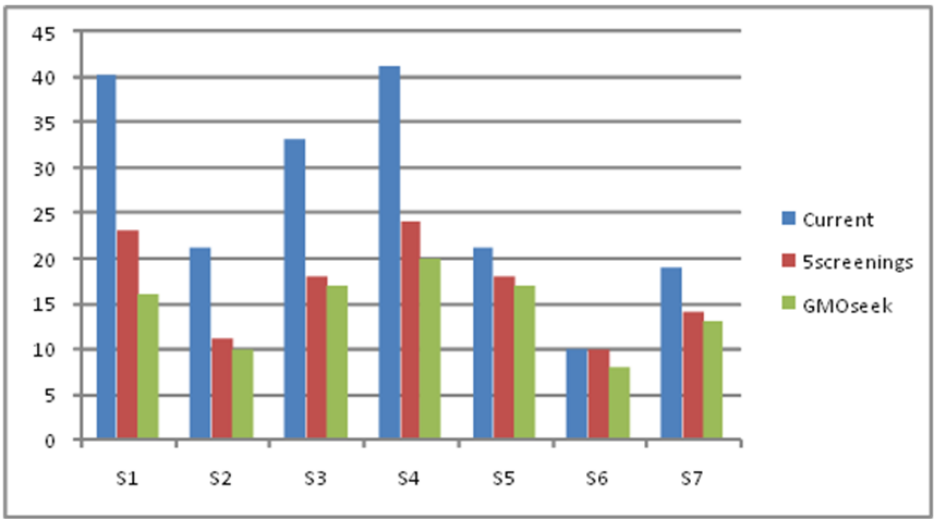

Figure 3: Number of tests needed for real routine sample analysis using different screening strategies

The tests were performed on seven real samples analysed at the NIB. The usual p35SxtNOS (+R73 for rapeseed) screening combination was compared to the five screening approach using the same methods proposed by Waiblinger and co-workers (Waiblinger et al. 2010) and to the optimal combination of screening methods proposed by the GMOseek algorithm. The Y axis represents the total number of qPCR tests (screening and event-specific) needed to conclude about the GMOs in the sample (see deliverable D3/01).

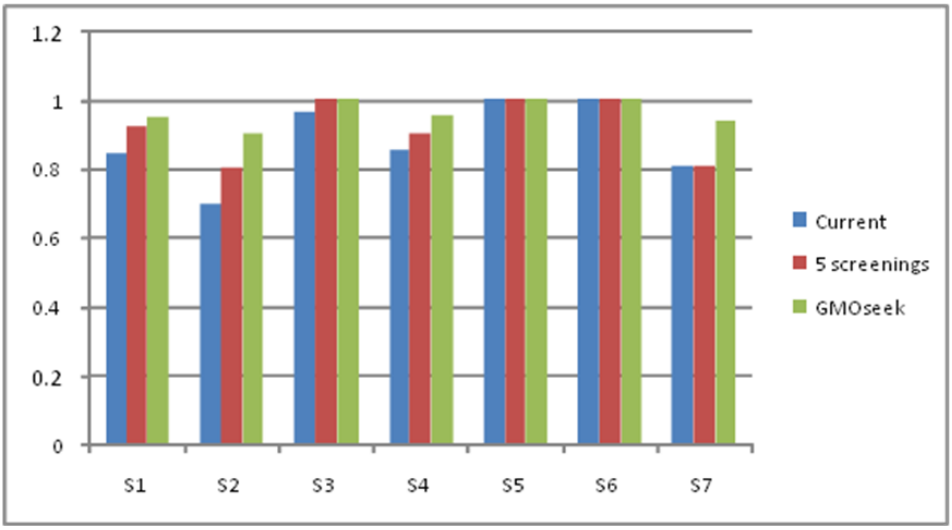

Figure 4: Percentage of GMOs covered for real routine sample analysis using different screening strategies

The tests were performed on seven real samples analysed at the NIB. The usual p35SxtNOS (+R73 for rapeseed) screening combination was compared to the five screening approach using the same methods proposed by Waiblinger and co-workers (Waiblinger et al. 2010) and to the optimal combination of screening methods proposed by the GMOseek algorithm. The Y axis represents the fraction of GMOs in the GMOmatrix covered by the different screening strategies (see deliverable D3/01).

Outputs for WP1

At the end of the two-year project period, WP1 has produced a verified GMOmatrix comprising 225 GMO references, and more than 240 genetic elements related to these GMO events. This matrix should be made available in the coming months to all interested people via publication in a peer-reviewed periodical. Furthermore, a bio-informatics tool named "GMOseek algorithm" has been developed to extract information regarding new screening methods to be developed for a screening step covering more GM events and for more cost-efficiency. The algorithm also includes a simple decision support system to help the analyst understanding the results of experimental screening tests and guide him through the whole GMO analysis. Another user-friendly excel-based tool has been developed to extract important information from the GMOmatrix. Both tools use a simplified version of the GMOmatrix as input. The matrix and its associated tools ease both the interpretation of analytical results and the decision of GM elements for the design of new singleplex and multiplex detection methods. It will be made freely available via request or on a website (e.g. dedicated page of GMOseek partners' websites) and can be used by different stakeholders including official authorities and private laboratories. The GMOmatrix produced during GMOseek is one of the sources used to built and verify the GMO database within the EUGINIUS project. An export module will be designed to generate updated matrix from the EUGINIUS database, compatible with the input GMOmatrix needed to run the GMOseek algorithm. This connection with the starting EUGINIUS project should ensure long lasting of the GMOseek results.

The concept of matrix-based approach as well as the bio-informatics tool (GMOseek algorithm) developed in this project are not limited to the European area but could be used elsewhere where GMO regulations exist. Moreover, they could also be adapted to other types of PCR-based detection assays (such as plant pathogen detection, veterinarian or even food health safety) for which different markers are use to screen the potential presence of targeted organisms in samples.

WP2: DNA-based amplification methods

With the growing number of GMOs being placed on the market, the need for more efficient detection methods is increasing. Therefore in WP2 the GMOseek project aimed at the development and the in-house validation of new screening methods that cover a wide range of GM events and that are time and cost saving. Additionally, the project planned to a second laboratory the in-house validated methods in order to evaluate their applicability, reproducibility and robustness.

In order to constitute convincing evidence for a pre-validation report, the in-house validation of all the methods was done taking into account the former French AFNOR experimental norm XP V03-020-2 (AFNOR 2003) (later re-qualified as application guide GA V03-020 (AFNOR 2006)) for the determination

of the limit of detection (LOD) and the recommended validation guidelines from the ENGL document "Definition of minimum performance requirement for Analytical Methods for GMO testing" (CRL-GMFF 2008) and the recent Codex Alimentarius guidelines on performance criteria and validation (Codex Committee On Methods Of Analysis And Sampling 2010).

Task 4: Novel singleplex screening methods

Subtask 4.1 -TaqMan® real-time PCR screening tests

Relevant appendices: D4.1/01: Singleplex TaqMan® methods: Standard Operating Procedures.; D4.1/02: Pre-validated Singleplex TaqMan® screening methods - Pre-validation report]

Three screening methods targeting genetic elements were developed and evaluated. These methods target the promoter pUbi from maize (*Zea mays*), the terminator tE9 from pea (*Pisum sativum*) and the 35S-*nptII* junction. The pUbi and tE9 genetic elements appear as interesting cost efficient screening targets for a better coverage of GMOs, according to the results of simulations using the GMOseek algorithm (see deliverable D3/01, and Table 1). **As the pUbi is originating from maize, this target is not suitable for GMO detection in products containing maize. For discrimination of GM rice or cotton containing this promoter, it must be checked if there is no trace of maize by using an endogenous target for this plant species.**

The pUbi, tE9 and 35S-nptII junction methods tested in this in-house validation reach the performance criteria of specificity, sensitivity, limit of detection, efficiency of amplification, rates of false positive and false negative results set by the EURL for quantitative methods (CRL-GMFF 2008) and indicated in deliverable D8/01. Robustness was also partially addressed. Results from in-house validation are available in the deliverable D4.1/01 (see appendix). These methods were transferred to a second laboratory for evaluation, the results of which are available in the deliverable D4.1/02 (see appendix). **The experiments performed in the second laboratory show that these methods are transferable. Additional tests should be performed according to the validation guidelines described in the deliverable D8/01** (in appendix). Summary of the tested methods performance is given in the Table 2**. If proving satisfying performances according to the criteria required in this document, these three methods will be ready for an international validation scheme.**

Two additional singleplex methods were developed and should be in-house validated at CRA-W before they can be considered for possible transfer and full-validation.

As the tE9 target originates from pea which can be found as ingredient in food and feed, it would be preferable if a control method for its plant of origin would be made available in order to verify if a positive results obtained with the tE9 method originates from the presence of a transgenic event or from the presence of pea in the sample. Four methods targeting endogenous pea genes were therefore developed and evaluated for specificity and sensitivity. **Although the verification of the performance is not completed, the lectin targeting method seems to be the best for pea detection and meets all the performance criteria so-far tested** (see Table 2 for a summary of the performance parameters so-far tested). The pea-specific method should be further in-house validated in order to evaluate if this method can be proposed for a full inter-laboratory validation. **As no transfer of the method was performed during the project, a pre-validation study including 2 to 4 labs would be recommended before entering a full validation**.

The *cry*1Ab gene from *Bacillus thuringiensis subsp. kurstaki* appears as an interesting target for better screening of GMOs, according to the results of simulations using the GMOseek algorithm (see deliverable D3/01 and Table 1). Furthermore, the GMOseek algorithm proposes that a combination comprising a method detecting *cry*1Ab would allow the detection of all the unauthorized GM rice events recently found on the EU market (KeFeng6, KMD1 and Bt63). As the cry genes are relatively variable even in the *cry*1Ab cluster, the method development first focused on its ability to detect *cry*1Ab variants present in various GM events. **Based on the preliminary results obtained during the project** (Table 2), **the *cry*1Ab specific method meets the performance criteria set during the project.** Given the importance of this screening method for GMO detection, **it would be beneficial if the in-house validation of the method targeting *cry*1Ab would be completed** in order to evaluate whether this method can be proposed for a full inter-laboratory validation. **As no transfer of the method was performed during the project, a pre-validation study including 2 to 4 labs would be recommended before entering a full validation**.

Subtask 4.2.- SYBR®Green real-time PCR method development within a CoSYPS format

[Relevant appendices: D4.2/01: SYBR®Green *methods: Standard Operating Procedure; D4.2/02: Upgraded COSYPS platform - Algorithm and manual instructions; D4.2/03: Pre-validated* SYBR®Green *screening methods -. Pre-validation report; appendix T35S]*

To enable the application of the patented 'Combinatory SYBR®Green PCR Screening' system (CoSYPS) (Van den Bulcke et al. 2010) for more GM events and to allow an extra discriminating power to this system, four new SYBR®Green screening methods were developed and validated at IPH according to the SOPs of the ISO 17025 accredited GMO platform.

The methods targeting pFMV, Cry3Bb and pNOS were in-house validated and successfully transferred to a second laboratory (see deliverables D4.2/01 and D4.2/03, in appendix)

These three methods are fit for their application in the routine detection of GM events in food and feed samples under ISO 17025. It was decided that they could be integrated into the CoSYPS matrix for routine GMO detection and their respective decision values could be introduced. The way the CoSYPS matrix is working and the introduction of the new real-time PCR screening methods was reported in deliverable D4.2/02 (in appendix). This deliverable also contains a short manual on how the CoSYPS matrix needs to be used for the analysis of routine food/feed samples.

The three SYBR®Green screening methods targeting pFMV, pNOS and Cry3Bb from IPH-GMOlab were developed and validated according to the in-house SOPs. The obtained results for the tested parameters are in accordance with these SOPs (see Table 2 for a summary of tested parameters). However to comply with the Deliverable D8/01, which was established recently, **more experiments need to be performed before the above mentioned methods can go to full validation**. The specificity tests have for example been conducted only on gDNA extracted from available wild type and GMO CRMs. More plant materials need thus to be gathered and tested. Additionally the $LOD_{95\%}$ and the robustness test need to be carried out. Except from the results from the already completed method transfer, **a pre-validation should be organised to be able to rate the false positive and false negatives**.

A fourth method targeting the t35S element has been developed but need further optimization before it is considered for possible transfer and full-validation. Details regarding this fourth method are given in the appendix t35S.

Subtask 4.3 –Evaluation of the screening element selection

[Relevant appendix: evaluation of the screening element selection*]*

The aim of this subtask was to experimentally test if the already existing screening elements are relevant by testing screening methods on the existing material, and to bring eventual corrections or implementation to the matrix in function of the results of the tests.

For this task, the CRA-W used the screening targets that were developed in the GMOSeek project (pUbi, tE9, cry1Ab) and the screening tests that were coming from previous CRA-W projects (pFMV35S, pRice Actin, pSSuAra, pTA29, t35S, tOCS, tg7, bar, EPSPS-1 and gox), from LGL (EPSPS-2) or from routine analysis (p35S, tNOS). More detailed results are presented in the appendix "Evaluation of the screening element selection".
The genetic element composition of 27 GM events, listed in the GMOmatrix, were experimentally verified. Information or partial information on 11 of these 27 events was found. Three events

(MON87705 maize, MON87769 maize and "J101, 103 alfalfa") contained the tE9 screening element and two events (86AB rice and MXB-13 cotton) the pUbi screening element. This further stresses the importance of these newly developed screening methods.
The importance of the newly developed screening methods may even be higher **but information regarding the composition of other events introduced in the matrix was sometimes scarce or non accessible. Moreover, this material was not available for testing. These two last points are general limitations when one wants to establish a database like our GMOmatrix and verify it with theoretical and experimental data.**

Task 5: Cost efficient implementation of methods: multiplexing PCR methods

[Relevant appendices: D5/01: 5-plex method: Standard Operating Procedure; D5/02: Pre-validated TaqMan® screening pentaplex - Pre-validation report]

LGL has developed a pentaplex real-time PCR targeting five screening targets (p35S, *tNOS*, the *pat*, *bar* gene and the CTP2-CP4EPSPS construct) which are frequent in authorized GM plants. The choice of the targeted screening elements in this pentaplex PCR was made based on the provisional GMOmatrix (WP1) and the data published by Waiblinger and collaborators (Waiblinger et al. 2010). According to the authors, **95% of the 42 tested GM events can be detected by methods targeting at least one of the p35S, tNOS, *bar* or CTP2-CP4EPSPS screening elements. The fifth element integrated in the pentaplex was the *pat* detection system as it allows a good coverage of GMOs according to our provisional GMOmatrix** (see WP1 and Table 1). Each of these candidate TaqMan® assays has either been published in at least one singleplex methods before (deliverable D5/01, in appendix). **As not every GMO laboratory has the equipment to perform a pentaplex real-time PCR method, a triplex (the p35S, tNOS, CTP2-CP4EPSPS) and duplex (*pat*, *bar*) real-time PCR have also been developed for these elements which have not been planned in the original DoW**.

After development and optimization, standard operating procedures (SOP) of the methods were written as a guideline for the in-house validation study and the transfer to second laboratory (deliverable D5/01, in appendix).

The pentaplex, triplex and duplex TaqMan® PCR assays were successfully in-house validated on several parameters, showing satisfying specificity, sensitivity, amplification efficiency (E), regression coefficient (R^2) and robustness according to official guidelines on singleplex assays as far as possible (CRL-GMFF 2008; Codex Committee On Methods Of Analysis And Sampling 2010) (detailed results in deliverable D5/02, in appendix). It is noteworthy that in addition to these usual performance parameters, **a stress test with extreme ratios in copy number between the analytic detection system and all others of the assay (asymmetrical LOD) was performed**. As the extreme conditions chosen are very unlikely to happen

in routine analysis the results of this stress test are satisfying. The asymmetrical LOD could not be determined as described in D8/01 because these guidelines were drafted later during the GMOseek project. Moreover doing it as requested by D8/01 requires working with targets sequences cloned in plasmids, which were not available at the time the validation was done. Such **asymmetrical LOD determination should therefore be repeated with plasmid as reference material.**

In addition to the in-house validation, **the three multiplex real-time PCR assays were successfully transferred** to CRA-W for statistical analysis of repeatability and reproducibility according to the ENGL criteria (CRL-GMFF 2008; Codex Committee On Methods Of Analysis And Sampling 2010). The pentaplex, triplex and duplex PCR assays demonstrated similar sensitivity, E and R^2 so they proved to be robust in the CRA-W laboratory on a LC480 instrument.

However, **the GMOseek consortium decided that the robustness should be validated following an orthogonal test scheme** (see deliverable D8/01, in appendix). **This elaborate robustness will need to be evaluated before a ring trial is envisaged for the pentaplex, triplex and duplex real-time PCR screening assays**. A summary of the so-far tested performance parameters for the three multiplex real-time PCR assays is given in Table 2.

Conclusions to WP2:

An important effort has been produced within the WP2 in terms of method development and in-house validation:

- ***three singleplex real-time PCR methods (targeting the elements pUbi, tE9, and the construct 35S-nptII) using TaqMan® probes technology were developed and in-house validated. Two additional singleplex real-time PCR methods (targeting the endogene lectin pea and the element cry1Ab) were also developed but more information about their performance criteria should be produced before they can be evaluated for full validation.***
- ***three multiplex real-time PCR methods (a duplex targeting the elements pat, bar, a triplex targeting the elements p35S, tNOS, CTP2-CP4EPSPS and a pentaplex (targeting the elements pat, bar, p35S, tNOS and CTP2-CP4EPSPS) using TaqMan® probes technology were developed and in-house validated.***
- ***four singleplex real-time PCR methods (targeting the elements pFMV, pNOS, Cry3Bb and t35S) based on the SYBR®Green technology were developed. The three first ones were in-house validated and the last one (targeting t35S) is presented in this final report (see appendix).***

All these qualitative real-time PCR methods add significant discriminatory power to the screening phase of GMO detection tests with increasing GMO coverage, higher chance to detect unauthorized GMOs and in a more cost efficient way. The five elements targeted by the multiplex methods cover all

but four GMO events that are authorized, tolerated or in pipeline for authorization in EU, as well as the unauthorized GMOs observed in EU (Table 1). These different methods were in-house validated under different international standards or recommendations discussed during the meetings of the GMOSeek project and taking into account the specificity, applicability, limit of detection and the false positive/negative rates. The methods were successfully tested for these performance parameters and successful transfers to a second testing laboratory provided evidence for their applicability in routine analysis.

During the last six months of the project, the deliverable D8/01 was produced based on different existing documents. It proposes guidelines for the validation of qualitative singleplex and multiplex methods including the performance parameters to be tested, practical procedures to do so, and the acceptance criteria for these parameters (see WP4). It is the opinion of the GMOseek consortium that the methods having proven their performance during the project should enter inter-laboratory validation studies following the D8/01 guidelines. To do so, in-house validation should be completed in accordance with these guidelines followed by the organization of pre-validation studies (involving a limited number – two to four- of laboratories). For methods successively passing the performance criteria and pre-validation studies, a full validation (involving several laboratories -12 to 15, figure to be discussed) could be performed as a prolongation of the GMOseek project. For each method developed within this WP2, the tested performance parameters and their suitability with the requirements proposed in the deliverable D8/01 are summarized in Table 2.

Table 1: "EU" GMOs covered by the qPCR methods developed within the GMOseek project

"EU" GMOs are the GMO events authorized (A), tolerated (T) or in pipeline for authorization (P) in the European Union. The events indicated in orange (U-EU), are the unauthorized GMOs for which presence was detected in the EU. In red are indicated the remaining four GMOs that cannot be detected with the screening methods developed within the GMOseek project and for which event-specific methods are necessary for detection.

GMOname	Status	specie	P-35s	T-nos	pat	bar	CTP2-CP4EPSPS	P-FMV	P-nos	T-35s	cry3Bb1	P-ubiZM1	T-E9	cry1Ab	35S-nptII	Sum targets	
Falcon GS/40/90	A/T/P	**canola**	1	0	1	0	0	0	0	1	0	0	0	0	0	**3**	
GT73 (RT73)	A/T/P	**canola**	0	0	0	0	1	1	0	0	0	0	1	0	0	**3**	
Liberator L62, pHoe6/Ac	A/T/P	**canola**	1	0	1	0	0	0	0	0	0	0	0	0	0	**2**	
MS1	A/T/P	**canola**	0	1	0	1	0	0	1	0	0	0	0	0	0	**3**	
MS8	A/T/P	**canola**	0	1	0	1	0	0	0	0	0	0	0	0	0	**2**	
RF1	A/T/P	**canola**	0	1	0	1	0	0	1	0	0	0	0	0	0	**3**	
RF2	A/T/P	**canola**	0	1	0	1	0	0	1	0	0	0	0	0	0	**3**	
RF3	A/T/P	**canola**	0	1	0	1	0	0	0	0	0	0	0	0	0	**2**	
T45 (= HCN28 = ACS-BN008-2) (≠ Topas 19/2)	A/T/P	**canola**	1	0	1	0	0	0	0	0	0	0	0	0	0	**2**	
Topas 19/2 (HCN92)	A/T/P	**canola**	1	0	1	0	0	0	1	0	0	0	0	0	0	**3**	
59122	A/T/P	**corn**	1	0	1	0	0	0	0	0	0	1	0	0	0	**3**	
87460	A/T/P	**corn**	1	1	0	0	0	0	0	0	0	0	0	0	0	**2**	
88017	A/T/P	**corn**	1	1	0	0	1	0	0	0	1	0	0	0	0	**4**	
89034	A/T/P	**corn**	1	1	0	0	0	1	0	0	0	0	0	1	0	**4**	*experimentally detected
176 (Bt 176) (b)	A/T/P	**corn**	1	0	0	1	0	0	0	1	0	0	0	1	0	**4**	
Bt10	U-EU	**corn**	1	1	1	0	0	0	0	0	0	0	0	1	0	**4**	
Bt11	A/T/P	**corn**	1	1	1	0	0	0	0	0	0	0	0	1	0	**4**	
Event 32	U-EU	**corn**	1	0	1	0	0	0	0	0	0	1	0	0	0	**3**	
Event 3272	A/T/P	**corn**	0	1	0	0	0	0	0	0	0	1	0	0	0	**2**	*experimentally non-detected
Event 98140	A/T/P	**corn**	1	0	0	0	0	0	0	0	0	1	0	0	0	**2**	
GA21	A/T/P	**corn**	0	1	0	0	0	0	0	0	0	0	0	0	0	**1**	
MIR162	A/T/P	**corn**	0	1	0	0	0	0	0	1	0	1	0	0	0	**3**	
MIR604	A/T/P	**corn**	0	1	0	0	0	0	0	0	0	1	0	0	0	**2**	
Mon810	A/T/P	**corn**	1	0	0	0	0	0	0	0	0	0	0	1	0	**2**	
Mon863	A/T/P	**corn**	1	1	0	0	0	0	0	0	1	0	0	0	1	**4**	
NK603	A/T/P	**corn**	1	1	0	0	1	0	0	0	0	0	0	0	0	**3**	
T25	A/T/P	**corn**	1	0	1	0	0	0	0	1	0	0	0	0	0	**3**	
TC1507	A/T/P	**corn**	1	0	1	0	0	0	0	0	0	0	1	0	0	**3**	
88913	A/T/P	**cotton**	1	0	0	0	1	0	0	0	0	0	1	0	0	**3**	
1445, 1698	A/T/P	**cotton**	1	1	0	0	1	1	0	0	0	0	1	0	1	**6**	
281-24-236	A/T/P	**cotton**	0	0	1	0	0	0	0	0	0	1	0	0	0	**2**	
3006-210-23	A/T/P	**cotton**	0	0	1	0	0	0	0	0	0	1	0	0	0	**2**	
GHB614	A/T/P	**cotton**	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	
LLcotton25	A/T/P	**cotton**	1	1	0	1	0	0	0	0	0	0	0	0	0	**3**	
MON 531	A/T/P	**cotton**	1	1	0	0	0	0	0	0	0	0	0	1	1	**4**	*experimentally detected
15985 (DB50xMON531)	A/T/P	**cotton**	1	1	0	0	0	0	0	0	0	0	0	1	1	**4**	*experimentally detected
FP967	U-EU	**flax**	0	1	0	0	0	0	1	0	0	0	0	0	0	**2**	
55-1, 63-1	U-EU	**papaya**	1	1	0	0	0	0	1	1	0	0	0	0	0	**4**	
AV43-6-G7	A/T/P	**potato**	0	1	0	0	0	0	0	0	0	0	0	0	0	**1**	
EH92-527-1	A/T/P	**potato**	0	1	0	0	0	0	1	0	0	0	0	0	0	**2**	
Bt63 (Shanyou, JinYou)	U-EU	**rice**	0	1	0	0	0	0	0	0	0	0	0	1	0	**2**	
KeFeng6	U-EU	**rice**	1	1	0	0	0	0	0	1	0	1	0	1	0	**5**	
LLRICE601	U-EU	**rice**	1	1	0	1	0	0	0	0	0	0	0	0	0	**3**	
LLRICE62	A/T/P	**rice**	1	0	0	1	0	0	0	0	0	0	0	0	0	**2**	
305423	A/T/P	**soybean**	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	
356043	A/T/P	**soybean**	1	0	0	0	0	0	0	0	0	0	0	0	0	**1**	
A2704-12, A2704-21, A5547-35	A/T/P	**soybean**	1	0	1	0	0	0	0	1	0	0	0	0	0	**3**	
A5547-127	A/T/P	**soybean**	1	0	1	0	0	0	0	1	0	0	0	0	0	**3**	
BPS-CV127-9	A/T/P	**soybean**	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	
GTS40-3-2	A/T/P	**soybean**	1	1	0	0	0	0	0	0	0	0	0	0	0	**2**	
MON87701	A/T/P	**soybean**	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	
MON87705	A/T/P	**soybean**	0	0	0	0	1	0	0	0	0	0	1	0	0	**2**	
MON87769	A/T/P	**soybean**	0	0	0	0	0	0	0	0	0	0	1	0	0	**1**	
MON89788	A/T/P	**soybean**	0	0	0	0	1	1	0	0	0	0	1	0	0	**3**	
H7-1, RUR H7	A/T/P	**sugarbeet**	0	0	0	0	1	1	0	0	0	0	1	0	0	**3**	
A5-15	A/T/P	**sugarbeet**	1	0	0	0	0	1	0	0	0	0	1	0	0	**3**	
Total number of target "hit"			**32**	**28**	**14**	**9**	**8**	**6**	**7**	**8**	**2**	**10**	**8**	**9**	**4**	4	Total number of non detected GMOs

Table 2: Summary table of the tested performance parameters and their suitability with the requirements proposed in the deliverable D8/01

Target: genetic element targeted by the method
Applicability: type of matrix tested during in-house validation
Specificity: OK means that no unexplained signal was observed on non target reference material. Tested on wild type and GMO reference materials.
LOD_6: absolute LOD (aLOD) determined according to the instructions detailed in the deliverable D8/01. The LOD_6 of a PCR run is the lowest target copies for which all six replicates in a PCR run gave positive results. Expressed in target copy number.
$LOD_{95\%}$: absolute LOD (aLOD) with 95% confidence determined according to the instructions detailed in the deliverable D8/01. Expressed in target copy number.
LOD_{20asym}: asymmetric LOD determined according to the instructions detailed in the deliverable D8/01. Expressed in target copy number and relative content (%).
Robustness: Orthogonal test to determine robustness of the method to various small changes in experimental conditions according to the instructions detailed in the deliverable D8/01. Partial robustness tests consisting in instrument change were performed for the multiplex methods.
False positive/false negative rates: To be determined during pre-validation and inter-laboratory studies according to the instructions detailed in the deliverable D8/01.

ND = not done
The performance parameters (efficiency, R2 coefficient, LOD6, LOD20asym) indicated in the table are averages of the data measured during in-house validation and transfer to second laboratory (when relevant). See the relevant deliverables (D4.1/01, D4.1/02, D4.2/01, D4.2/03, D5/01, D5/02) for further details about methods' performance. Only for the amplification efficiency of multiplex methods, data are given for individual tested cyclers and laboratory ($^{\Delta}$ Stratagene MX3005P at LGL, $^{\copyright}$ Roche LC480 at LGL, $^{\#}$ Roche LC480 at CRA-W)

Real-time PCR technology	Target	Applicability	Practicability	Specificity	LOD_6 (target copy number)	$LOD_{95\%}$	Robustness (orthogonal test)	False positive/ false negative rates
SYBR®Green	**pNOs**	gDNA (from CRM) and pDNA	Methods implemented in routine lab + methods transferred without problem	OK	1-3	ND	ND	ND
	pFMV				13-26	ND	ND	ND
	***cry*3Bb**				6-12	ND	ND	ND
	t35S	gDNA (from CRM)	ND		2-19	ND	ND	ND
Singleplex Taqman®	**pUbi**	gDNA (from CRM) and pDNA	Methods transferred without problem	OK	20	ND	ND	ND
	tE9				2	ND	ND	ND
	P35S-*nptII*	gDNA (from CRM)			16	ND	ND	ND
	lectin (pea)	gDNA from non certified plants	ND	OK	<20 but more repetitions are necessary	ND	ND	ND
	***cry*1Ab**	gDNA (from CRM) and pDNA				ND	ND	ND

Real-time PCR technology	Target	Applicability	Practicability	Specificity	Amplification efficiency	R^2 coefficient	LOD_6	$LOD_{95\%}$	Asymmetric limit of detection (LOD_{20asym})	Robustness (orthogonal test)	False positive/ false negative rates
Multiplex Taqman®	P35S	gDNA (mix of CRM)	Methods transferred without problem	OK	97%-107%[Δ] 99%-103%[☺] 111%-115%[#]	98%-100%	<10	ND	≤ 0.035% (20 HGE)	Partial robustness tests OK	ND
	TNOS				90%-93%[Δ] 96%-109%[☺] 91% - 115%[#]	99%-100%	<20	ND	≥1.93%(1,080 HGE)	Partial robustness tests OK	ND
	CTP2-CP4-EPSPS				93%-101%[Δ] 119%-121%[☺] 111%-117%[#]	99%-100%	<5	ND	≤ 0.036% (20 HGE)	Partial robustness tests OK	ND
	pat				95%- 104%[Δ] 91%-99%[☺] 92%-99%[#]	99%-100%	<20	ND	≤ 0.035% (20 HGE)	Partial robustness tests OK	ND
	bar				100%-109%[Δ] 96-112%[☺] 109%-112%[#]	95%-100%	<10	ND	≥ 0.11% (60 HGE)	Partial robustness tests OK	ND

WP3: DNA-based hybridization methods

[relevant appendices: D6/01: NAIMA platform - Standard Operating Procedure; D6/02: Pre-validated NAIMA screening platform - Pre-validation report]

Within this work package, the goal was to establish an alternative to PCR-based methods for GMO detection. For this, NIB has developed further the NAIMA amplification system in combination with microarray detection (Morisset et al. 2008) for multiplex screening of GMO. NAIMA is an alternative DNA-based amplification technology to PCR. It was first developed within the Co-Extra European project and has shown to allow multiplex, sensitive and fast amplification with quantitative aspects for GMO detection. The task for NIB was to optimise NAIMA in order to demonstrate the application of this new technology to support multiplex screening of GMOs (as a complement to new real-time PCR methods) as well as for the detection of unauthorized GMOs harbouring known genetic elements.

A deliverable (D6/01, in appendix) details the NAIMA optimisation and in-house validation phase during the project.

The experimental verification of the hexaplex assay performed on genomic DNA dilution series and food/feed samples confirmed the good sensitivity and specificity of the assay regarding NAIMA multiplex amplification. The **microarray-based detection of NAIMA products gave good results in terms of sensitivity but false-positive signals were observed**, some of them probably due to cross-reactivity of the capture probes. However, EV ILVO was trained before transfer to this laboratory for the NAIMA hexaplex assay in order to gather further data regarding the multiplex NAIMA amplification performance and microarray-based detection of NAIMA products to fully evaluate the method's performance**.** Details about the transfer results are available in the deliverable D6/02 (in appendix).

Conclusion for WP3:

The NAIMA amplification showed good performance if only qualitative analysis is needed and the amplification method could be transferred successfully to EV ILVO. However, for one of the six amplicons, the method is not robust to unequal target concentrations (Mon810 target at low level, the others at high level). This problem should be solved before the multiplex amplification can be used as it affects the sensitivity of the assay and could lead to false-negative results at low target concentration.

The transfer to EV ILVO failed to provide further information regarding the performance of NAIMA product detection on microarray. None of the microarrays hybridized at EV ILVO were suitable for data analysis. Moreover, analysis of the microarrays hybridized at NIB has shown that the detection of NAIMA products on microarray is not suitable for GMO detection although sensitivity on microarray was satisfactory. The use of high-density micro-array is hampered by the lack of

repeatability/reproducibility of the platform, the need for trained personnel, the high cost of equipment and reagents, and the time to get results (due to the long hybridization and the different washing steps). Therefore, we conclude that this method should not go to further validation step before the robustness of NAIMA amplification to unequal target concentration is proven, and that appropriate detection platform (low cost, high speed, specificity and sensitivity) is not proposed.

WP4: Reference material and guidelines for validations

Task 7: Production of reference materials

The aim of task was to make available genomic DNA (gDNA) extracted from (certified) reference materials ((C)RM) to all project partners in support of the WP2 and WP3 activities.

Additionally, plasmids had to be produced for each selected real-time PCR method and to be registered under 'Safe Deposit' collection of the BCCM (Ghent, BE).

Subtask 7.1.- In-house plant RM production and certification

To fulfil this subtask, a list of all available reference materials was made at IPH. This list contained eight species (maize, soybean, oilseed rape, cotton, sugar beet, potato, papaya and rice) and 41 GM events (including five stacked events) (see deliverable D7/01, in appendix). This valuable information helped to decide which materials should be made available to develop the new screening methods described in the respective work packages (WP2, WP3).

Four different plant species (maize, soybean, oilseed rape and potato) were chosen comprising each time the wild type (WT) material and at least one GM event. This resulted in a total of four WT materials and six GM events to be made available for all partners in the GMOseek project.

The extraction and characterization of the materials was done as described in deliverable D7/02 (in appendix). Materials were tested for precision, purity and status of inhibitory effect on amplification.

End of June 2010, the **requested materials were distributed by IPH to LGL and CRA-W All characterization information concerning the distributed materials was sent to the respective partners.**

Subtask 7.2.- Plasmid reference production and certification

For each selected real-time PCR method developed and validated, a corresponding plasmid containing the targeted amplicon in a pCR2.1 or pUC18 background had to be produced and sequenced. Each partner had to produce the plasmid corresponding to the method(s) they develop if needed. Subsequently the plasmids have to be registered under "Safe Deposit" at the BCCM collection (Ghent, BE).

The production of the plasmids was as reported in deliverable D7/02 (in appendix).

At IPH, three of the four needed plasmids have been constructed in a pUC18 background, namely pFMV, Cry3Bb and pNOS and are available in a pUC18 background. **For all methods developed at CRA-W a plasmid reference material is available in a pCR2.1 or pUC18 background**. They were tested along with the gDNA using the newly developed real-time PCR methods (in WP2). A scientific dossier was established and the plasmids will be deposited at the BCCM collection in 2011. **The construction of the t35S marker plasmid is delayed at IPH** as the development of the method is problematic (see appendix t35S and deliverables D4.2/01 and D4.2/03 in appendices). **The construction of plasmids for the methods developed at LGL is on-going**. For the multiplex methods, plasmids harbouring each individual amplicon but also one plasmid harbouring all targeted sequences need to be constructed for determination of the asymmetric LOD.

Conclusion for WP4, T7:

The extracted gDNA materials were successfully produced, and characterised. They were used for the in-house validation of screening methods as outlined in WP2. By extracting gDNA from ten different materials, performing an intensive characterization of it and distributing the requested materials to the partners in time, subtask 7.1 was successfully fulfilled by IPH.

By constructing and characterising plasmid reference material for each of their developed and validated methods, IPH and CRA-W fulfilled subtask 7.2. At CRA-W, the constructed plasmids still need to be sequenced and deposited. The construction of plasmids for the methods developed at LGL is on-going.

As the development at IPH of the screening method for the fourth target (t35S) is on-going and some additional experiments are needed, the construction of the corresponding plasmid is delayed. When the method will function in an optimal way, it will be in-house validated and the plasmid will be prepared. All plasmids will be deposited at the BCCM collection (Ghent, BE).

Task 8: Preparation of validation guidelines

The Taq®Man and SYBR®Green real-time PCR screening methods and the NAIMA hybridisation platform needed to be in-house validated and transferred to a second laboratory (WP2 and WP3). As no guidelines on requirements for validation of qualitative methods exist, the GMOseek partners needed to establish such a document based on official guidelines (such as IUPAC, ISO, Codex Alimentarius) and filling gaps were needed.

It was later decided that the validations should be performed according to the newly established Codex Alimentarius document (Appendix III of report CAC/GL 74-2010) to be able to eventually introduce the developed and validated methods under ISO. The deliverable D8/01 "Validation Guidelines. Report" (in appendix) was submitted on 13/04/2011.

The GMOseek consortium fulfilled task 8 by elaborating guidelines for the validation singleplex and multiplex real-time PCR methods for the purpose of qualitative screening of GMOs. This document will further be used to evaluate the newly developed methods within the project. Estimation will be done for each method to decide if more work needs to be performed before the method can go to a full validation through a comparative study. This will form the basis of a discussion with FSA for a possible prolongation of the project. Furthermore, the document was distributed to the German §64 LFGB GMO working group. The document was also introduced to the European Network of GMO laboratories working group on Minimal Performance requirements (in which Dany Morisset actively participates) which is currently working to update the document "Definition of minimum Performance requirements for analytical methods of GMO testing" used as guidance document for all GMO applicants in the European Union. The updated version should include requirements for qualitative (screening) methods as well as multiplex methods, thus relevant to the output of the D8/01.

Conclusions

The GMOseek project has successfully fulfilled its initial objectives by the production of many methods and tools that should help enforcement laboratories inside and outside the EU improving the detection of authorized and unauthorized GMOs.

A **simple comprehensive excel table named GMOmatrix** gathering the genetic elements introduced in GMOs was compiled and thoroughly verified. Part of this information was also experimentally verified within the project, when practical. The use of this GMOmatrix is supported by **two bio-informatics tools** build to help the analyst choosing the **best screening strategy**, **find genetic elements** that should be targeted by new screening methods to be developed, **guide the analyst** throughout the full detection process to identify possible GMOs in a sample and **suggesting the potential presence of stacked events and/or unknown unauthorized GMOs**. This GMOmatrix and the bio-informatics tools will be made freely and openly available to all interested public, shortly after the project completion. When the export tool of the EUGINIUS project will have been developed (by the end of 2011), it will still be possible to use the updated matrix generated from this database with the bio-informatics tools from the GMOseek project. This should ensure a **long lasting usability** of the tools developed in the project. **Regarding the results of the bio-informatics activities, it is recommended to set-up a website in which the GMOmatrix and the GMOseek algorithm, optimised for web interface, would be freely and constantly available to all potential users.** This matrix approach and its associated bio-informatics tools could also be adapted to other domain of detection where screening methods are needed. Additional efforts to collect data about GMOs and implement the GMOmatrix would be needed. Therefore, contacts were already made with Chinese (Dr Litao Yang, Shanghai Jiao Tong University, Shangai) Japanese (Dr Katzumi Kitta, National Food Research Institute, Ibaraki) and Indian (Dr Gurinder Jit Randhawa, National Bureau of Plant Genetic Resources, New Delhi) to collaborate on such **extension of the GMOmatrix scope**.

Nine singleplex and three multiplex real-time PCR methods were developed by partners of the GMOseek project. **Most of them have been in-house validated and transferred** to a second laboratory to evaluate their practicability. Furthermore, used in combination with other screening methods, the singleplex methods **add significant information** to the screening phase results, helping to discriminate which GMO event(s) may be present in a sample. The multiplex methods developed in this project allow an **almost full coverage of all authorized GMOs in EU**, as well as the non-authorized events found in EU. All these methods showed high sensitivity to detect very trace of targets in the sample, and great specificity to the target (see Table 2). **Most of the real-time methods** developed during GMOseek (the singleplex Taqman® real-time PCR targeting pUbi, tE9, P35S-nptII, and lectin –pea-, the SYBR®Green real-time PCR targeting pNOS, *cry3*Bb and pFMV, the three multiplex Taqman® real-time PCR methods) **showed very good performance suitable with routine use in GMO detection**. They were also proven to be **important in terms of the range of GMOs (authorized and UGM) they cover** (multiplex methods) and

in terms of the discriminatory power they bring for the final GMO identification (all methods). **It is therefore recommended that these methods are quickly made available to the enforcement laboratories after being fully validated**. Still, **some additional tests will be needed** to fully assess the performance of these methods. **None of the methods went through orthogonal robustness test or determination of the $LOD_{95\%}$.** For some of the methods, specificity should be verified on additional target and non-target material (if practical). **Finally, the LOD_{20asym} should be assessed on plasmid material for the multiplex real-time PCR methods.** When these last parameters will have been tested and shown to be in accordance with the criteria set in D8/01, the method should enter a pre-validation step (involving two to four laboratories) followed (if the method performs properly) by a full inter-laboratory trial study. **For the Taqman® real-time PCR targeting the *cry*IAb gene and the SYBR®Green real-time PCR targeting the t35S element, further optimisation and some more experimental verification tests are needed.** Given the importance of both methods, it would be preferable that they are also made available to all enforcement laboratories, probably once they will have been optimised and their performance fully characterised.

To support the further validation of the qPCR methods, the partners of the GMOseek project have produced **guidelines describing the parameters one should measure to verify the performance of a screening method**. These guidelines will be followed to complete the in-house validation of the methods as well as for the pre-validation and inter-laboratory trials. Moreover, these guidelines were already distributed to the European Network of GMO laboratories formed by almost 100 national enforcement laboratories, via the working group on Method Performance Requirements. These guidelines will be one of the sources used to update already existing official guidelines that are currently into re-evaluation at the ENGL. It is also expected that these guidelines are beneficial to laboratories in other domains than GMO detection, where PCR-based methods are needed (e.g. food microbiology, environmental testing...).

The activities on the NAIMA amplification procedure combined with detection on microarray were not satisfying. Although the NAIMA amplification showed very promising performance for higher multiplexing, it was also limited by a lack of sensitivity when one of the targets is present a low concentration, while other targets are in high concentration. Moreover, the microarray-based approach failed to provide reliable detection; some GMOs present in samples remaining undetected. Therefore, this approach was considered as not practical and not satisfying for GMO detection unless significant improvement is made.

As illustrated in the following table, the activities in GMOseek required much more efforts in terms of personnel time (Table 3) and material than it was initially planned. This is due for example to the addition of multiplex methods to the initial DoW (LGL in WP2) so that laboratories without the proper equipment for pentaplex method can still use multiplex methods for high throughput analysis. Also, CRA-W produced an additional bio-informatics tool for the use of the GMOmatrix (WP1). NIB decided

to upgrade the initial GMOseek program to a user-friendly interface so it is easier to use the algorithm (WP1). This increase in activities led to additional efforts in terms of finances. This additional cost was covered by the GMOseek partners own resources. It is a general opinion within the consortium that the funding obtained from the SAFEFOODERA platform (by far the least funded of the SAFEFOODERA projects) was not sufficient in regards to the importance of the issue to develop better GMO screening strategies and methods, and in regards of the provided results. The GMOseek partners have nevertheless made substantial efforts to complete the project as they believe it is highly relevant to the Food Safety issue.

Table 3: Staff efforts during the GMOseek project

Partner organisation	Contact person	Person month (PM) initially planned	Person month (PM) at the end of the GMOseek project	Person month (PM) in last 6 month period (from 1st of November 2010 to 31st May 2011)
NIB	Dr Dany Morisset	8.8	12.4 (+41%)	3.2
CRA-W	Dr Gilbert Berben	11.4	19.52 (+71%)	5.84
EC JRC-IHCP	Dr Marc Van den Bulcke	0.9	2.25 (+150%)	1.25
EV ILVO	Dr Isabel Taverniers	2.0	3.16 (+58%)	1.56
IPH	Dr Nancy Roosens	16.1	22.43 (+39%)	11.17
LGL	Dr Ulrich Busch	18.0	32.0 (+78%)	8.4

A problem encountered during the project and linked to the matrix-based approach is the fact that **some certified reference genomic DNA materials (CRMs) are contaminated with other GM events** than the one(s) for which they are certified. For example, during the activities of T7.1 it was noted that not all purchased materials were completely pure and that unexpected screening markers were positive in the

CoSYPS. This was already seen previously in several GMOseek partner laboratories when gDNA extracted from CRMs, to be used for method development and in-house validation, was screened. It must be reminded a CRM is certified for the presence (and content) of a certain GM event but not for the absence of contaminating events. The origin of this contamination is not known, however it is generally observed that contaminations in CRMs are trace contaminations (very weak signals are observed for the markers of the contaminating GM event) and that the contaminating GM event is authorized in the EU. For example, IPH notes that the unexpected markers that were found positive in CRMs always showed a Ct value that was at least 10 Cts higher than the main ingredient (respective endogene) and than the expected markers.

The use of contaminated CRMs for the method development and validation is not problematic and does not influence the results if the genomic DNA is used as a positive control. For positive control, the use of plasmid DNA harbouring the target screening sequence is also a good alternative. However, if genomic DNA needs to be used as a negative control, the use of contaminated CRMs could bias the analysis of the method development and validation results. When possible, **specificity tests (negative controls)** during development and validation of new screening methods **should be done using non-contaminated CRMs**. Otherwise, the contaminated CRM(s) should be fully characterised to anticipate possible non-expected positive signals when used as negative control. In case the contamination may interfere with the negative control analysis, the CRM should not be used. For details regarding this matter are available in the deliverable D7/02, in appendix.

The contaminated CRMs are also problematic when one needs to experimentally verify the GMOmatrix, as observed during the task T4.3 (see appendix evaluation of the screening element selection). Due to the restricted availability of CRMs, it is almost impossible to obtain an alternative to a contaminated CRM. Therefore, the GMOmatrix needs to be experimentally verified with great care, bearing in mind this issue. Prior to the verification, it is preferable to test the CRMs for finding and identifying possible contamination. This way, positive signals for the contaminant-associated markers can be anticipated. Generally, much weaker signals will be observed for these markers due to the trace amount of the contaminating GM event(s). It is then relatively straightforward to fill-in the GMOmatrix with experimental data when the source of contamination is known. In case that the contaminant is not known, a clear difference in signal between expected and unexpected markers can be also used to differentiate the screening markers linked to the GM event in study, from the makers linked to the contaminant; and therefore it is still possible to fill in the GMOmatrix. It is only in the case of a reference material (not certified) contaminated with high content of an unknown GM event that it may not be possible to experimentally verify data in the GMOmatrix. This case does not apply to authorised GMOs (or GMO in pipeline for authorisation) in EU that always have CRM available to the routine laboratories. Therefore, **contamination of reference material with other GM event is posing problems only for unauthorized GM events** for which little information is known regarding their associated reference

material. Given the difficult access to unauthorised GM material, this last case is not of high relevance for the project.

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Appendices

GMOseek Part 2: Appendices 1-4

Appendix 1: D1.01 (Model GMO matrix. Report)

Appendix 2: D3.01 (GMOseek algorithm analysis performed. Report)

Appendix 3: D4.1.01 (Singleplex TaqMan® methods: Standard Operating Procedures)

Appendix 4: D4.1.02 (Pre-validated Singleplex TaqMan® screening methods. Pre-validation report)

GMOseek Part 3: Appendices 5-12

Appendix 5: D4.2.01 (SYBR®Green methods: Standard Operating Procedure)

Appendix 6: D4.2.02 (Upgraded COSYPS platform. Algorithm and manual instructions)

Appendix 7: D4.2.03 (Pre-validated SYBR®Green screening methods. Pre-validation report)

Appendix 8: D6.01 (NAIMA platform: Standard Operating Procedure)

Appendix 9: D6.02 (Pre-validated NAIMA screening platform. Pre-validation report)

Appendix 10: D7.01 (List of Reference Material already available for methods development and validation)

Appendix 11: D7.02 (Reference Materials available for methods pre-validation. Report)

Appendix 12: D8.01 (Validation guidelines)

GMOseek Part 4: Appendices 13-14

Appendix 13: D5.01 (Cost efficient implementation of methods: Pentaplex PCR/Duplex PCR/Triplex PCR. Standard Operating Procedures.)

Appendix 14: D5.02 (Cost efficient implementation of methods: Pre-validated TaqMan® screening pentaplex PCR/Duplex PCR/Triplex PCR. Pre-validation report.)

GMOseek Part 5: Appendices 15-21

Appendix 15: Scientific papers (Published scientific papers associated with this project)

Appendix 16: T35S (Development and validation of a SYBR®Green real-time PCR method targeting the t35S element)

Appendix 17: Screening element selection (Evaluation of the screening element selection)

Appendix 18: Communication (Communication about the GMOseek project activities)

Appendix 19: Project participants (Persons having participated to the project)

Appendix 20: Deliverables and milestones (Report on deliverables and milestones)

Appendix 21: Problems_challenges_amendments (Problems/challenges/amendments)