

JRC TECHNICAL REPORTS

Detection, Interpretation and Reporting on the presence of authorised and unauthorised genetically modified materials



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Detection, Interpretation and Reporting on the presence of authorised and unauthorised genetically modified materials

European Network of GMO Laboratories (ENGL)

The working group and its mandate

The working group was established on the basis of a mandate adopted by the ENGL Steering Committee. The working group has been chaired by Ilaria Ciabatti (Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis, Italy) and Patrick Philipp (Service Commun des Laboratoires, Strasbourg, France).

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The mandate of the Working Group was the following:

1. Review the document "Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified material" published in 2011 in particular broadening the scope to authorised GMOs and to uncovered taxa, taking into account recent observations and reports on the RASFF and technological developments.
2. Starting from Figure 2 of the above-mentioned document, provide practical guidance to improve and facilitate harmonisation for detection, interpretation and reporting for authorised GMOs, unauthorised GMOs and GMOs that fall under Regulation (EU) No 619/2011.
3. Review available information and technological developments, identify gaps and provide guidance on how these can be closed, taking cost-benefit aspects into consideration. In case during its activity the WG identifies methods suitable to fill existing gaps, these may be forwarded to the ENGL Advisory Group on Selection of Methods for Validation (AG SMV).

4. Discuss pros and cons of targeted (knowledge-based) versus non-targeted (unbiased) approaches to GMO detection. In this respect, a targeted approach can be understood e.g. as an analytical strategy based on information on the origin/composition of the sample (specific country, crop or inserted element or construct).
5. Provide guidance on recommended approaches for verification of analytical results for GMO detection, with clear indications of requirements and status as evidence.
6. Set up a priority of actions and list identified unclosed gaps.

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1. Introduction

Genetically modified organisms (GMOs) and products derived thereof have been commercialised on the food and feed market for more than twenty years. Many countries in the world have introduced legislative requirements for the authorization, traceability and labelling of GMOs, but the regulatory framework may differ from country to country. Various factors, including commercial interests, drive the request of developing companies for the authorisation and marketing of GMOs in specific areas of the world. This leads to the situation where a certain GM event may have different authorisation statuses in different countries (what is sometimes referred to as “asynchronous GMO approval”).

EU enforcement laboratories have to verify that no unauthorised GM product is entering the EU market; they also have to check that authorised GM products are properly traced and labelled (Regulation EC 1829/2003, Regulation EC 1830/2003, Directive 2001/18/EC, Regulation EU 619/2011). This requires that validated qualitative screening methods for GM elements and constructs plus qualitative and quantitative event-specific methods are available for application on a large variety of food and feed matrices ranging from raw materials, including seeds, to highly processed products. Real-time PCR has proven to be the most reliable and effective methodology and thus it has become the method of choice for GMO detection.

In many cases, unless the Competent Authority specifies a targeted analytical request, enforcement laboratories adopt a non-targeted approach where routine GMO testing is designed to provide an answer to the following questions: “Is there a GM event in the sample?”, then “Which GM event(s) is (are) present in the sample?”, “Is (are) this (these) event(s) authorised in Europe?” and in case of authorised GM event(s) identified, the question asked will be: “What is the percentage of the GM event(s)?”. In order to answer these questions, the laboratory usually implements a stepwise analytical work flow:

1. detection of the ingredient(s)/component(s)/constituent(s) of the sample: this is performed using taxon-specific methods
2. GMO screening: this is usually performed using element and/or construct-specific methods
3. GM event identification using qualitative event-specific methods
4. GM event quantification using quantitative event-specific methods

Given the variable complexity of the matrices to be analysed and considering the ever-increasing number of GM events to be detected, the need for a screening phase which can detect all the potential GM events and, at the same time, steers the subsequent analytical steps towards a subset of targets depending on the results of the screening tests, has been widely recognised. In particular, the so called “matrix approach”, already described in the European Network of GMO Laboratories (ENGL) guidance document “Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials” (2011) and in CEN/TS 16707:2014, is considered to be the most powerful and practicable approach currently available for improving cost-effectiveness and reliability of GMO analysis.

A number of laboratories in Europe have been already using a matrix approach for screening purposes, although little harmonisation exists in this regard. Differences may be related to the type and number of genetic elements/constructs targeted and/or to the detection method used for a specific target. Depending on a number of factors (e.g. the type of food/feed matrix, the origin of the product, the plant species present in the sample, etc.), the laboratory should be given the freedom of choosing the most suitable set

of screening targets for the intended purpose. Nonetheless it would be recommended to adopt common practices in the use of screening matrices, as this would enhance harmonisation of the GMO screening approach in the EU.

Another approach for efficient testing is provided by the use of pre-spotted plates, where a large number of authorised and possibly unauthorised GM events are simultaneously screened for.

Powerful tools for the application of the matrix approach and the pre-spotted plates are described in this document and some guidelines are provided.

The stepwise approach adopted in routine testing is supported by a decision tree, where a Yes/No (or Detected/Not detected) result drives the following course of action. Method performance is certainly an essential parameter to be verified to ensure reliable test results and this is currently carried out in the EU according to harmonised guidelines (ENGL Guidance documents “Definition of Minimum Performance Requirements for analytical methods of GMO testing” and “Verification of analytical methods for GMO testing when implementing interlaboratory validated methods”). However, verification and interpretation of the analytical result can also affect the reliability of the laboratory’s test response. This document is intended to address this issue, providing guidelines for the verification and the interpretation of analytical results. References to international standards (in particular ISO standards) with recommended criteria for reporting are also provided.

As specified above, usually GMO testing is carried out using a non-targeted approach, which means that available information on the type and origin of the product to be checked is not considered in the definition of the testing strategy. Although representing the only choice when such information is not available, not relevant or very limited, this approach has a number of drawbacks: considering the ever-increasing number of GM events marketed worldwide, with a diversification of crops, biotech traits, genetic elements and constructs introduced, the non-targeted approach requires the implementation of an extensive set of methods, including screening, event identification and quantification methods, which makes GMO testing very costly and increasingly time consuming. Furthermore, from a more general perspective, taking into account that the official control, carried out according to Regulation (EC) No 882/2004, can perform physical checks, including analytical testing, only on a limited fraction of the food or feed marketed in the EU, a non-targeted approach may be inefficient, giving many negative results, at least in food testing, rather than identifying non-compliances to current regulations. Given this context, the present guideline presents the concept of the so called “knowledge-based approach”, which can be described as a product-centred approach based on Web mining and knowledge discovery technology, followed by analytical confirmation. The document will consider different types of information that can be used and an analysis on how the approach could contribute to the efficiency and the effectiveness of the subsequent GMO detection strategy.

Finally the guideline provides an overview on current uses and future perspectives of the use of Next Generation Sequencing technologies applied to GMO testing.

2. Definitions and acronyms

ENGL: European Network of GMO Laboratories

EURL GMFF: European Union Reference Laboratory for GM food and feed

GMO method matrix: a relational presentation (e.g. a table) of symbols or numbers, where one dimension (e.g. column) corresponds to genetic elements and genetic constructs detected by a defined PCR method and the other dimension (e.g. row) corresponds to GM events. The entered symbols or numbers indicate the detectability or non-detectability of the target sequence for the GM event.

GMO target matrix: a relational presentation (e.g. a table) of symbols or numbers, where one dimension (e.g. columns) corresponds to genetic elements or genetic constructs present in a GMO and the other dimension (e.g. rows) corresponds to GM events. The entered symbols or numbers indicate the presence or absence of the target for the GM event and copy number, if available.

LOD : Limit of Detection

LOQ : Limit of Quantification

NGS: Next Generation Sequencing

NRL: National Reference Laboratory

U: Expanded Measurement Uncertainty

MRPL: Minimum Required Performance Limit

UGM: Unauthorised Genetically Modified Organism

3. Screening for genetic elements, constructs or GM events

3.1 The matrix approach

3.1.1 Background

Globally the number of different genetically modified organisms (GMO) and the use of products containing GMO is constantly increasing. Thus the analysis for GMO in a given sample has become progressively complex and elaborate. To employ only event-specific methods when testing for GMO presence is not very efficient. Therefore, alternative detection strategies have to be applied. A considerable number of scientific papers and recommendations have been published which depict strategies that have been developed for the identification of GMO. The most time and cost efficient strategy to detect a potential GMO in a sample is to use a screening approach which employs the combination of element-specific detection methods selected on the basis of analytically proven or theoretical information on the presence or absence of the target (specific DNA sequence) in a list of GMOs followed by event-specific methods (if available) [<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>].

A European Technical Specification (CEN/TS 16707:2014) for the application of PCR-based screening strategies using the so called matrix approach is available. According to this document a 'GMO method matrix' or, alternatively, a 'GMO target matrix' can be applied (see definitions). Examples for the implementation and application of the matrix approach are described. In order to ensure reliable analytical results, the document also provides guidelines for the validation of the performance of qualitative PCR methods applied in the screening approaches.

In addition, the principles and the application of the matrix approach are also described in detail in the document "Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials" elaborated by the European Network of GMO Laboratories (ENGL) ad hoc working group on "unauthorised GMOs" (European Network of GMO Laboratories, 2011a).

Different available tools for applying the matrix approach for GMO analysis and for developing specific screening strategies are described in the following section.

Until today there is no harmonized procedure for applying the matrix approach. Each of the tools described in the following chapter can be helpful to meet the specific needs of the GMO testing laboratory. Nevertheless the quality (experimental proof vs. theoretical information), completeness and update of the data are important aspects when choosing between the different tools. These tools are basically intended to support competent authorities and other users dealing with complex GMO analysis.

3.1.2 Tools for application of the matrix approach

Screening Table

The German laboratory network developed a 'GMO method matrix', which is based on a specific set of methods targeting the most frequently present genetic elements and constructs (Waiblinger et al., 2010).

The set currently comprises eight TaqMan real-time PCR methods for detection of the.

- Cauliflower mosaic virus (CaMV) 35S promoter (P-35S)
- nos terminator derived from *Agrobacterium tumefaciens* (T-nos)
- ctp2-cp4epsps junction of the chloroplast-transit peptide (CTP2) from *Arabidopsis thaliana* and the epsps gene from *Agrobacterium tumefaciens* strain CP4 (cp4-epsps)
- bar gene from *Streptomyces hygroscopicus*
- P-35S-pat junction of the CaMV P-35S promoter and the synthetic pat gene
- promoter from Figwort mosaic virus (P-FMV)
- modified cry1Ab/Ac gene from *Bacillus thuringiensis* (cry1Ab/Ac)
- nos promoter from *Agrobacterium tumefaciens* (P-nos).

All methods have been validated in collaborative trials, are included in the GMOMETHODS database of the EURL GMFF (Bonfini et al., 2012) and are published in the Annex of the ISO 21569 standard or as ISO Technical Specifications. Available reference materials are used for verification of the data set presented in this method matrix.

The matrix is maintained by the German National Reference Laboratory (NRL-GMO). The complete verification dataset of the 'Screening Table' is available as MS Excel table or on-line in the EUginus database (see below). In addition, a list of available GMO reference materials can be downloaded from the internet or is searchable on-line in EUginus.

Links:

[Screening Table GMO](#) (Version 2015)

[Reference Materials](#) (Version 2016)

[EUginus Verification Table](#) (Version 2016)

[EUginus Reference Materials](#) (Version 2016)

GMOseek software and GMOseek matrix

The GMOseek software, developed in European ERA-NET GMOseek project, is designed to support decision making in all the phases of routine GMO laboratory testing, including the interpretation of wet-lab results (Morisset *et al.*, 2014). The tool makes use of a tabulated matrix of GM events and their genetic elements, the laboratory analysis history and the available information about the sample at hand. The tool uses an optimization approach to suggest the most suited screening assays for the given sample. The practical GMOseek user interface allows the user to customize the search for a cost-efficient combination of screening assays to be employed on a given sample. It further guides the user to select appropriate analyses to determine the presence of individual GM events in the analyzed sample, and it helps taking a

final decision regarding the GMO composition in the sample. GMOseek can also be used to evaluate new, previously unused GMO screening targets and to estimate the profitability of developing new GMO screening methods.

The advantage of GMOseek software is its adaptability to new situations on the market by changing the input data matrix, which includes data about GMOs, methods (assays) for detecting GMOs and probabilities of GMO presence. The software is publicly available and can be downloaded from the project's website (<http://www.gmoseek.com/gmoseek>).

The same internet page hosts also:

- the comprehensive 'GMO target matrix', from Block and collaborators also developed under the framework of the European ERA-NET GMOseek project, adapted to GMOseek software. This matrix provides a comprehensive and user-friendly overview of 273 genetic elements and their occurrence in 328 GMO (Block *et al.*, 2013).
- another matrix with data on GM events approved in the EU as well as the events regulated under Regulation EU 619/2011 to be used directly with the GMOseek program. These datasets can be further edited in commonly used spreadsheet programs (like OpenOffice Calc or MS Excel) and saved into a tab-separated file.

GMOfinder

The *GMOfinder* is a combination of both a 'GMO target matrix' and a 'GMO method matrix' (Gerdes *et al.*, 2012). It is based on an MS Access database with implemented algorithms and for example facilitates the interpretation of the outcome of screening analyses. The underlying comprehensive tabular matrix, where each GMO is assigned to its specific profile of 15 detectable and non-detectable genetic elements and constructs, lists more than 360 GMO events from 29 plant species (May 2016). Information on selected genetic elements and constructs derives from experimental data, literature, applications for GMO authorisation and other web sources. The *GMOfinder* offers an optional inclusion of events with potentially masked genetic elements in the results listing. This is especially important when analysing mixed profiles due to the presence of more than one GMO in a sample. Special care was taken to record the sources and the quality of the information (e.g. theoretical vs. experimental data), thus facilitating careful evaluation of screening results. Additionally, datasets can be semi-automatically exported to MS Word to record the changes over time. The database is maintained and updated regularly. The *GMOfinder* is available on request free of charge from the corresponding author (sven.pecoraro@lgl.bayern.de).

CoSYPS matrix

The 'combinatory qPCR SYBR-Green screening' (CoSYPS) approach is a patented GMO target matrix-based decision-support system for interpretation of analytical results (van den Bulcke *et al.*, 2010). It is considering the presence of the following genetic elements in GMOs: the CaMV 35S promoter and terminator, the nos promoter and terminator derived from *Agrobacterium tumefaciens*, the FMV promoter, the *nptII* gene from *Escherichia coli*, the *epsps* gene from *A. tumefaciens* CP4, the *epsps* gene from *Zea mays*, the *pat* gene from *Streptomyces viridochromogenes*, the *bar* gene from *S. hygrosopicus*, the barnase

gene from *Bacillus amyloliquefaciens* and several *cry* genes (*cry1Ab*, *cryAc*, *cryF*, *cry3Bb*) from *B. thuringiensis* and more recently pCambia t35S (Fraiture *et al.*, 2014). The system is modular and any method can be developed and added at any time when necessary. The GMO element-specific methods were combined with SYBRGreen taxon-specific PCR methods targeting the important plant species. By applying the whole set of methods and through application of a “prime number”-based algorithm, CoSYPS indicates which GMOs are possibly present in a sample. The application of the CoSYPS platform and approach was validated in an inter-laboratory trial (Barbau-Piednoir *et al.*, 2014)

GMO Checker

This screening application from Japan which was developed as a TaqMan real-time PCR array is a 'GMO method matrix' (Mano *et al.*, 2009). The platform can be used for the comprehensive and semi-quantitative detection of genetically modified crops. It is a combination of 14 event-specific and 10 element specific methods. The specificity and sensitivity of the PCR assay were evaluated experimentally and are tabulated in the publication. As a tool to support the implementation of the approach, an Excel spreadsheet application for the evaluation of analytical results concerning the presence of LM crops has been developed and can be downloaded from the internet.

http://cse.naro.affrc.go.jp/jmano/UnapprovedGMOChecker_v2_01.zip

Extended Element Screening Approach

A broad element screening using 15 TaqMan real-time PCR methods (P-35S, T-nos, P-FMV, cp4-epsps(1), cp4-epsps(2), *cry1Ab*, *cry1Ac*, *cry3Bb1*, *cry1F*, *pat*, *bar*, *nptII*, rice actin 1 gene intron, barnase and barstar) has been described by RIKILT Wageningen UR (Scholtens *et al.*, 2013). The matrix given in this publication is a «GMO method matrix» with methods that are experimentally verified against a large set of reference materials. The broad screening strategy described promises to reduce the number of subsequent identification analyses, particularly for feed samples and can also give indications for unauthorized GMOs, if not all elements can be explained by subsequent event-specific tests. A table displaying the specificity of the methods against the set of reference materials is given in the publication. The GMO analysis tool of the EUGenius database (see below) is foreseen for easier evaluation of the screening results.

The set of available screening methods is under constant development and has now been updated with the TaqMan-based assays for detection of the *cry1Ab/Ac* element (Grohmann, 2015), ctp2-cp4epsps construct (Grohmann *et al.*, 2009), *cry1A.105* and *cry2Ab2* elements (Dinon *et al.*, 2011) plus the element screening methods described by Debode *et al.* (2013). The actual number and combination of methods in the screening set can be chosen from the available methods and can be optimized for the set of samples to be analyzed.

EUGenius

The EUGenius web-based GMO database provides an integrated application for user-defined 'GMO method matrices' (www.eugenius.eu). EUGenius is based on the 'European GMO Initiative for a Unified Database

System' and is an initiative of BVL and RIKILT in order to support competent authorities and private users, who seek accurate information on GMOs with a focus on the situation in the EU as well as world-wide coverage. The database provides several web-tools to plan and/or interpret the analysis of sample material. One of the search tools called 'Detection' gives access to experimental verification data of PCR screening methods: information on the specificity of a selected PCR method and its ability to detect GMOs is provided, e.g. the data of the 'Screening Table' and of the 'Extended Element Screening Approach'. A user-defined set of screening methods can be combined with several filtering functions. The resulting 'GMO method matrix' then displays the verification data (based on *in silico* or experimental specificity tests) for the selected detection methods.

In addition, when starting the search, a specific set of screening methods (Waiblinger *et al.*, 2010) can be preselected. Besides the method-related search tools mentioned above, the database provides a search option to check the source and availability of GMO reference materials.

In addition, a GMO analysis tool is accessible. The user enters the chosen methods and the outcome of the analysis ('detected' or 'not detected' results for the targets) and an interpretation is provided. When detected targets can be explained by detected events, no further action will be necessary. When targets are not explained by the detected events, a table with suggested output will list GMOs that could explain the detected targets and analysis results.

The EUginius database is available on-line at the internet address www.euginius.eu with a dataset that will be constantly increased and updated.

JRC GMO-Matrix

The JRC GMO-Matrix is a 'GMO method matrix' developed by the Joint Research Center's EURL GMFF (Angers-Lousteau *et al.*, 2014) that exclusively presents *in silico* results from simulations of PCR amplification and, when applicable, probe binding using bioinformatics tools, such as 're-PCR' (Rotmistrovsky *et al.*, 2004) and 'matcher' (Rice *et al.*, 2000). The methods available are those from the EURL GMFF's GMOMETHODS database (Bonfini *et al.*, 2012). The event sequence information used for the bioinformatics analyses is found in the Central Core DNA Sequences Information System (CCSIS), a local database that stores annotated GMO event sequences either retrieved from public sequences databases or submitted to the EURL GMFF as part of the GMO authorization procedures (Patak, 2011). The JRC GMO-Matrix currently includes more than 80 single events and more than 100 reference methods. The JRC GMO-Matrix relies on a relational database that contains pre-computed values corresponding to the extent of matching between the methods primers and probe and each GMO sequence, ranging from 0 (no amplification detected) to 2 (amplification detected with perfect annealing of both primers and probe). An intermediate score of "1" is shown when a potential amplicon has been detected, despite imperfect binding of the primers and/or probe (up to an arbitrary threshold of maximum 2 gaps and 2 mismatches per primer). The database is updated every time a new method or event sequence becomes available. The requirement for the event's sequence information limits its capacity to incorporate newly developed GMO events for which this information is not readily available. However, once an event sequence is determined, or a new detection method incorporated in the GMOMETHODS database, the fact that the JRC GMO-Matrix

is based on *in silico* predictions allows to rapidly populate all the additional cells of the matrix without the need for extensive laboratory testing.

The current version of the JRC GMO-Matrix application contains two interfaces, that are expected to grow based on the feedback that will be received from the user.

These interfaces are:

GMO events/Detection method matrix

This interface can be used for building two-dimensional matrices (chosen GMO events vs. chosen GMO methods) in order to visualize the universal coverage of the detection methods and identify potential gaps.

GMO Event finder

This interface allows identification of potential GMO(s) present in the sample based on a set of experimental positive and negative detection method results.

The JRC GMO-Matrix is freely accessible on the website of the EURL GMFF, at the address <http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/>

3.2 Pre-spotted plates

Pre-spotted plates are ready-to-use plastic supports for real-time PCR in which a mixture of reagents is dispensed in advance. Such tools are customised in terms of support type, number of assays and reagents dispensed (oligonucleotides only or complete reaction mixtures) and can be delivered frozen, air-dried or lyophilized.

They present the advantage of being time- and cost-efficient, while offering an easy-to-use straightforward tool to face high-throughput testing needs. Once the DNA is extracted, the operator only needs to prepare a single reaction mixture to be loaded into the plate in order to perform a multi-target analysis in a single PCR experiment. This approach reduces drastically the laboratory workload in terms of procurement and preparation of reagents, analytical testing and the associated risk of errors.

The first application of pre-spotted real-time PCR plates for the detection of GMOs was developed by the Joint Research Centre (JRC) of the European Commission (Querici *et al.*, 2009) with the aim of facilitating and harmonizing GMO testing in the European legal context. The pilot application included 48 TaqMan real-time PCR assays, most of which derived from the event-specific reference methods validated by the EURL GMFF and the ENGL.

In 2008 the feasibility of using pre-spotted plates was evaluated in collaboration with the ENGL on 389 real samples by 31 laboratories (data not published) resulting in a broad coverage of food and feed matrices. Kluga *et al.* (2012) also demonstrated that it allowed the detection of low amount of GMOs, therefore confirming the applicability to processed food matrices. Since the first application different layouts have been described in both 96-well and 384-well plate format, in single and duplex real time PCR assays (Mano *et al.*, 2009; Gerdes & Pecoraro, 2009, Gerdes *et al.*, 2011, Cottenet *et al.*, 2013) .

The major drawbacks when using pre-spotted plates are the lack of flexibility in testing scheme and the necessity to obtain high quality DNA in considerable amount. Also, the increasing number of GMO introduced in the market calls for periodical updates of such a support, and, as a consequence, introduces a challenge of space limitation. For example, the first 96-well/48 assays layout (Querici *et al.*, 2009) provides a valid solution to test in parallel two extraction replicates per sample, as requested by ISO 24276 standards, but the requirement to test simultaneously positive and negative controls represents a challenge due to space limitation. In order to comply with the necessity to verify the absence of false positive and false negative results, a periodical control of functionality of each lot of pre-spotted plates was implemented. This also allows verifying that the signals obtained in case of amplification are comparable over time.

Taking into account the above-mentioned bottlenecks, as well as the results of a survey conducted within the ENGL, the EURL GMFF has undertaken the development of a novel GMO screening 96-well pre-spotted plate. This new tool will offer the possibility to screen for the presence of all possible GMOs listed in the EU register of authorised GMOs, combining 16 taxon-, element-, construct- and event-specific assays, thus decreasing both the workload and the amount of DNA material needed. A dedicated web-based decision support system was made available to interpret the outcome by using a matrix providing a list of GM events that might be present in the analysed sample.

The EURL GMFF is also exploring the possibility to use the pre-spotted plates for semi-quantitative GM content evaluation. Such an improvement would further reduce the workload by allowing the testing

laboratory to simultaneously perform the detection and semi-quantification of known GMOs, therefore avoiding the quantification step for those samples in which only a weak contamination of authorised GM events is detected. A first approach was described in Kluga *et al.* (2012), but further investigations are necessary to confirm the reliability and transferability of this approach.

3.3 Checking for donor organisms

A number of screening assays are based on the detection of elements frequently used in genetic engineering, such as promoter, terminator, generic coding or marker gene sequences. Positive samples are further analysed for identification and quantification of specific GM event. Detection of a screening element in a sample with no event being detected may occur in the following situations:

- The screening method has a lower LOD than the event-specific method(s) and/or the screening element is present in multiple copies: this becomes relevant when the sample contains one event or multiple events at very low levels and cannot be solved unless introducing more template DNA in the reaction;
- The sample contains unknown events or events for which no detection method is available; in this case other strategies may be implemented (see e.g. European Network of GMO Laboratories, 2011a and last chapter of this guidance);
- The native donor organism of the detected screening element or its DNA is present in the sample. In this case the donor organisms may be identified with methods targeting sequences other than the screening element.

The last case is further addressed below. None of the methods listed below are expected to detect all strains of CaMV, FMV or *Agrobacterium*, because single nucleotide polymorphisms may exist between the different strains in the primer and probe binding sites. This means that in case of a negative test result, the presence of a donor organism still cannot be 100% excluded.

3.3.1 Cauliflower mosaic virus (CaMV)

The majority of GM plants on the market today have been transformed with the CaMV 35S promoter. Screening for the 35S promoter is therefore a powerful tool to detect GM plants. However, the source of 35S promoter could also be naturally occurring CaMV. Samples containing viral DNA can therefore be misinterpreted as positive to GMO detection.

PCR methods detecting native CaMV are based on the recognition of virus DNA sequences outside the promoter region. A number of methods are available and have been published.

One method detects the CaMV inclusion body matrix or reverse transcriptase gene using conventional PCR (Wolf *et al.*, 2000).

Cankar *et al.* (2005) have developed a real-time PCR method that detects virus coat protein gene sequences in the sample, allowing distinction between virus infected and genetically modified plants. However, as CaMV genes encoding capsid proteins are successfully used in generating genetically modified plants with increased tolerance to viral infection (e.g. Jaccaud *et al.*, 2003), these gene sequences might be used also in

the future in generating GM plants. Therefore, ability of this method to detect solely native infection of CaMV may be questionable in the future.

Chaouachi *et al.* (2008) selected the small CaMV protein gene involved in virus infectivity as a target to detect and quantify native CaMV contamination with real-time PCR.

3.3.2 Figwort mosaic virus (FMV)

Instead of commonly used 35S promoter, several new GM plants contain the 34S promoter from FMV. As the number of plants harbouring this type of promoter is increasing, it has become reasonable to include FMV 34S promoter in the screening matrix. Since certain plant species, e.g. *Scrophularia californica* (California figwort or California bee plant) are susceptible to FMV contamination, the ability to verify the screening result might be very useful. A method to detect native FMV was developed by Moor *et al.* (2012). This method targets a non-conserved coding region of FMV open reading frame VII.

3.3.3 *Agrobacterium tumefaciens*

Promoter and terminator sequences of *Agrobacterium* nopaline synthase gene (*nos*) are commonly used in GM plants. Methods detecting especially the terminator sequence of the *nos* gene are widely used for screening purposes in GM analyses. A real-time PCR method developed by Weller *et al.* (2002) and targeting an *Agrobacterium* chromosomal DNA sequence coding for a flagellar switch protein, detects a number of *Agrobacterium* strains and can be used as tool to identify native *Agrobacterium* contamination.

3.3.4 Reference material

Reference materials to be used as positive controls in the test for donor organisms are available from the following sources:

- ATCC (<http://www.lgcstandards-atcc.org/>)
- Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures: DSMZ Collection of Plant Viruses. Accessed via <http://www.gbif.org/dataset/86403148-f762-11e1-a439-00145eb45e9a> on 2014-10-28

A plasmid positive control to be used in association with the method of Cankar *et al.* (2005) for CaMV detection, was developed by Burns *et al.* (2013).

4. Interpretation of results and reporting

4.1 Purpose

This chapter is designed to help the laboratory:

- to take the appropriate decisions on compliance on the basis of the results obtained and the requirements of the EU legislation on GM food and feed currently in place;
- to provide all the relevant data and information on analytical reports according to the obligations of the EU legislation on GM food and feed and ISO standards.

This part of the document is not exhaustive, it intends to present the most frequent cases and it has to be considered as an assistance. Some particular cases may need more adapted interpretation.

The analytical approach and the rules for interpretation to be followed for the detection of unauthorised GM rice originating from China (for the implementation of Decision 2013/287/EU) are not considered in this section, as they are provided in a EURL GMFF specific guidance (EURL GMFF, 2014).

This chapter should also be modified according to possible future developments and/or legal requirements (e.g. new tolerance or labelling thresholds, etc.)

4.2 Scope

This chapter applies to the official analytical control of the following GM events for the enforcement of EU legislation on GM food and feed:

- GM events authorised on the EU market for which a 0.9% traceability and labelling threshold applies, provided that their presence is adventitious or technically unavoidable: the laboratory is requested to quantify the presence of these events.
- GM events whose authorisation is pending or expired in the EU, which are falling under the scope of Reg. (EU) 619/2011: the laboratory is requested to verify the presence of these GM events taking into consideration the Minimum Required Performance Limit (MRPL) set by the Regulation at 0.1%. A technical guidance document on the implementation of Commission Regulation (EU) 619/2011 has been developed and published by the EURL GMFF (2011).
- GM events withdrawn from the EU market, to which a Commission Implementing Decision on the withdrawal from the market and on a tolerance period for traces applies (e.g. Ms1, Rf1, Ms1xRf1, Rf2, Ms1xRf2, TOPAS 19/2 swede rape for which a tolerance threshold at 0.1% applies until 31/12/2016): the laboratory is requested to verify the presence of these GM events taking into consideration the tolerance threshold.
- GM events unauthorised or withdrawn from the EU market, which are not falling under the scope of Reg. (EU) 619/2011 and to which no Commission Implementing Decision on the withdrawal from the market and on a tolerance period for traces applies: the laboratory is requested to verify the presence of these GM events without any tolerance threshold or any MRPL.

This chapter is not addressing the official analytical control of seeds, for which no traceability/labelling threshold or MRPL has been established in the EU. However the reporting requirements are also applicable to seed testing.

4.3 Legal basis

- Regulation (EC) No 1829/2003 on genetically modified food and feed.
- Regulation (EC) No 1831/2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC.
- Commission Regulation (EU) No 619/2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.
- Commission Implementing Decision 2012/69/EU of 3 February 2012 amending Decisions 2007/305/EC, 2007/306/EC and 2007/307/EC as regards the tolerance period for traces of Ms1xRf1 (ACS-BNØØ4-7xACS-BNØØ1-4) hybrid oilseed rape, Ms1xRf2 (ACS-BNØØ4-7xACS-BNØØ2-5) hybrid oilseed rape and Topas 19/2 (ACS-BNØØ7-1) oilseed rape, as well as of their derived products (notified under document C(2012) 518).
- Authorized GMOs in the EU:
http://ec.europa.eu/food/dyna/gm_register/index_en.cfm

4.4. Interpretation and conclusions in the analytical flow

The decision tree presented in Figure 1 is referring to the following stepwise analytical work flow, which is generally adopted by laboratories to verify the enforcement of EU legislation on GM food and feed:

1. detection of the ingredient/component/constituent of the food/feed: this is performed using taxon-specific methods
2. GMO screening: this is usually performed using element and/or construct-specific methods
3. GM event identification using qualitative event-specific methods
4. GM event quantification using quantitative event-specific methods

However, there may be specific cases where one or more of the steps listed above are not necessary or useful and may be skipped. For UGMs step 4 will usually not be possible because of the lack of reference materials, anyhow zero tolerance of UGMs is prescribed in EU.

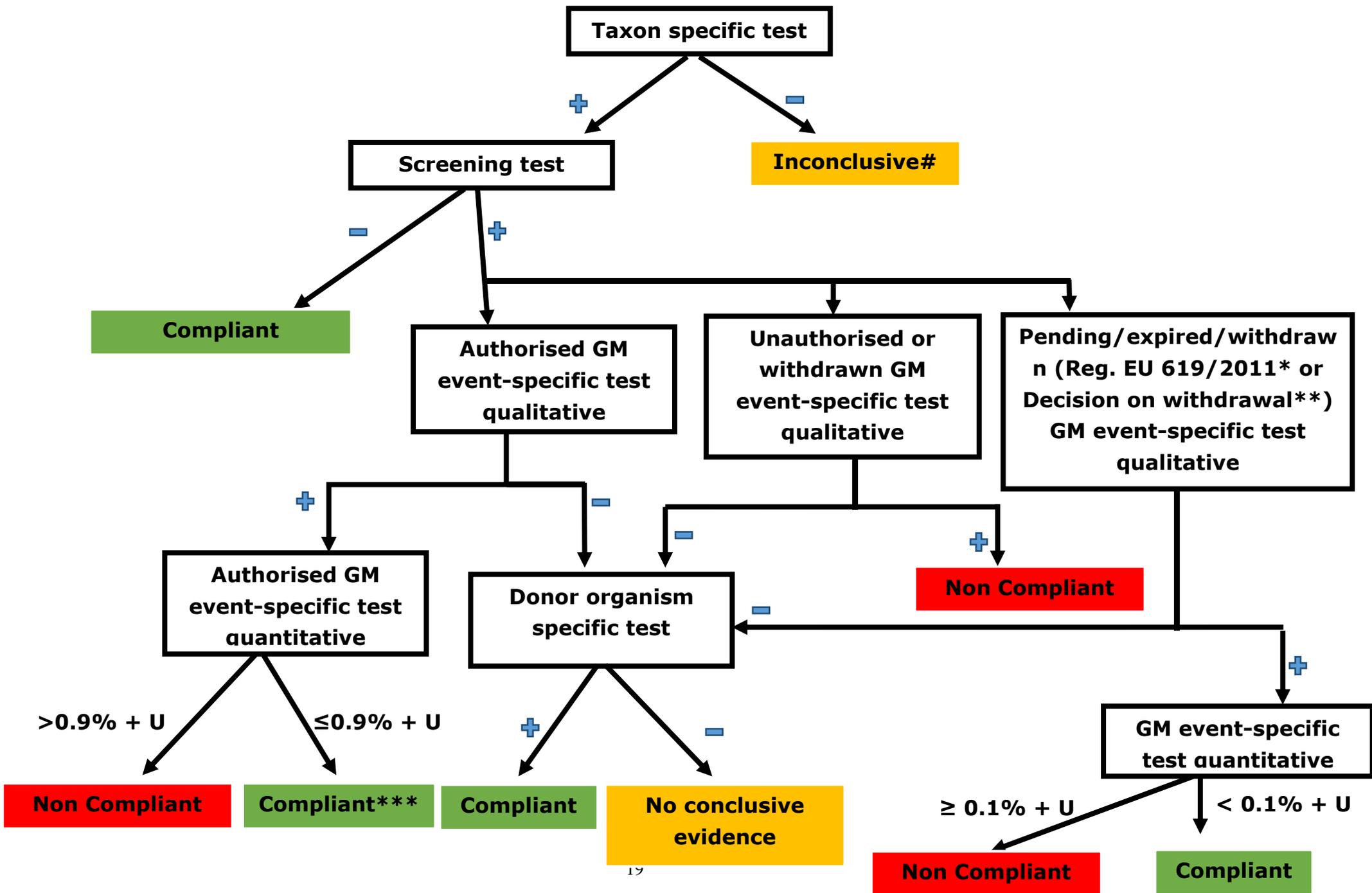


Figure 1 - Decision tree referring to the stepwise analytical work flow, which is generally adopted by laboratories to verify the enforcement of EU legislation on GM food and feed

The term "inconclusive" is used in this context, with the meaning that, if a taxon is not detected, the analytical request to detect and identify possible GM events for this taxon cannot be satisfied.

* 0.1% Minimum Required Performance Limit applies to methods for the detection of GM events falling under the scope of Regulation (EU) No 619/2011

** A tolerance threshold applies to some products according to specific Commission Implementing Decisions on their withdrawal from the market: e.g. Commission Implementing Decision 2012/69/EU of 3 February 2012 amending Decisions 2007/305/EC, 2007/306/EC and 2007/307/EC as regards the tolerance period for traces of Ms1xRf1 (ACS-BN004-7xACS-BN001-4) hybrid oilseed rape, Ms1xRf2 (ACS-BN004-7xACS-BN002-5) hybrid oilseed rape and Topas 19/2 (ACS-BN007-1) oilseed rape, as well as of their derived products; it sets a 0.1% tolerance threshold until 31/12/2016.

*** provided that this presence is adventitious or technically unavoidable

Results should be reported according to ISO 24276/Amd1:2013, ISO 21569/Amd1:2013, ISO 21570/Amd1:2013.

Table 1 summarizes the requirements set in ISO standards for the test report:

REPORT	ISO 17025	ISO 21569	ISO 21570	ISO 21571	ISO 24276
Title	x				
Lab's name and address	x				
Unique identification of the test report	x				
Customer's name and address	x				
Identification of the method used	x		x	x	
Identification of the CRM used			x		
Date of the sampling	x				x
Description/condition/unambiguous identification of the sample	x				x
Any particular information relative to the sample (insufficient size, degraded state...)					x
Size of the laboratory sample and size of the test portion				x	x
Date of receipt of the sample when critical	x				x
Storage conditions (if necessary)					x
Date of performance of the test	x				x
Reference to the sampling plan	x				
Tests results with units of measurement	x				x
Name, function and signature of person(s) authorizing the test report	x				
Name, function and signature of person(s) responsible for the analysis				x	x
A statement to the effect that the results relate only to the item tested	x				
Where necessary for the interpretation of the test results					
Deviation from, additions to, or exclusions from the test method	x			x	
A statement of compliance/non compliance with requirements/specifications	x				x
A statement on the estimated U	x				x
Opinions and interpretations	x			x	
Any additional information which may be required	x				
For test reports containing the results of sampling					
Unambiguous identification of the substance, material or product sample	x				
Location of sampling, diagrams, sketches or photographs	x				
Reference to the sampling plan and procedures used	x				x
Details of any environmental conditions during sampling that may affect...	x				
Standard, specification, deviations, additions or exclusions from the specification	x				
OPINIONS AND INTERPRETATIONS + COMMENTS					
Shall be clearly marked	x				
e.g. opinion on the statement of compliance/noncompliance	x				
Reference to ISO 24276 (compliance of the report to ISO 24276)			x	x	x
EXPRESSION OF THE RESULTS					
Negative results		x	x		x
Positive results		x	x		x
LOD of the method		x			x
LOD of the sample					x
LOQ of the method		x			
LOQ of the sample		x			
Specificity of the analytical method		x			
SUB CONTRACTORS					
Results clearly identified	x				

Table 1 - Requirements set in ISO standards for the test report.

5. Knowledge-based approach

5.1 The current strategy for GMO detection

As reported in the Introduction, currently GMO detection occurs, in the majority of cases, via a non-targeted approach. However, certain classes of UGMs are intrinsically difficult to detect using current molecular analytical strategies for reasons outlined in the ENGL ad hoc working group on “unauthorised GMOs” report and a publication (European Network of GMO Laboratories, 2011a; Holst-Jensen *et al.*, 2012).

In this chapter, we propose a paradigm shift in the way UGMs can be discovered: a product-centered approach based on Web mining and knowledge discovery technology, followed by analytical confirmation. This approach could also be adapted for steering the analyst to a priori detection and characterization of the authorized GMOs in a sample. But it could also assist the Competent Authority in developing the most efficient monitoring plan.

5.2 Shortcomings of present strategies for GMO and UGM detection

The current GMO testing strategy shows several shortcomings when aiming at the detection of UGMs. First, UGM detection based on molecular analytical technology depends on a near-exact match between the product and a predefined list of markers that identify (any or all) potential UGMs. Because the design of an analytical method requires prior knowledge of the identity and the molecular structure of the UGM, products that are unknown to authorities are generally excluded from the detection (among the exceptions are genetically homogenous products like a papaya fruit where detection of screening elements from the fruit may be sufficient to declare it as genetically modified)

Second, the cost and complexity of the current screening-based approaches will continue to increase due to further diversification of biotechnology traits on global markets (EURL GMFF Status of dossiers, International Service for the Acquisition of Agri-biotech Applications (ISAAA) GM Approval Database, Center for Environmental Risk Assessment GM Crop Database). A significant increase in efficiency is essential to accommodate comprehensive and economically acceptable UGM screening.

Third, the common sampling schemes cover only a fraction of the market due to limitations in testing resources and capacity, and selection of products relies mostly on random sampling of the food chain or particular product categories instead of targeted selection of suspect products. Consequently, the probability of a rare UGM event entering the testing laboratory is low. Taken together, resources are predominantly spent on confirming the absence of a predefined list of markers, and not on the targeted confirmation of suspect products.

Fourth, when UGM detection is based on screening for “common” markers, unambiguous recognition of a UGM is negatively influenced due to the compositional complexity of the sample. For instance, the presence of an authorized GMO ingredient or the presence of a botanical impurity being an authorized GMO, can mask the presence of an UGM in mixed foodstuffs.

In the opposite, a positive observation of an “expected” marker provides direct evidence for the presence of a UGM, but the choice of markers to test for is far from obvious and the list can be virtually unlimited. Together, this leads to the undesirable situation that unauthorized, but nevertheless marketed, products may remain undetected using the current monitoring and detection approach. The less is known about a UGM (e.g. not officially registered in order to obtain an authorization for food/feed use), the more difficult it will be to assess its safety, the higher the perceived risk, and the more difficult (unlikely) it will be to detect it by the currently applied standard analytical methods.

5.3 Off the beaten track: a product-centred approach could be a possible solution for UGM discovery and an adaptation towards a more efficient GMO detection

A novel, product-focused approach based on Web science technology (i.e. using Web search together with data mining and knowledge discovery technology) (Ruttink *et al.*, 2010a) followed by analytical confirmation (“product-to-sequence”) (Ruttink *et al.*, 2010b), provides an alternative to the current screening approach, which is based on analytical methods followed by deduction of the authorization status of the product (“sequence-to-product”) (Figure 2). In retrospect, most reported UGM incidents were initially discovered due to targeted searching for information on products, some readily available in the public knowledge domain. In several independent cases, detailed analyses of global trading records revealed that GMO products were being imported into regions where they were not authorized (Made *et al.*, 2006; Greenpeace GM Contamination Register). For instance, by 2000 approximately three quarters of the papaya production in Hawaii was based on GM papaya that was engineered to provide resistance against Papaya ringspot virus, and which was not approved in the EU. Based on this knowledge, the enforcement authority of Bavaria (Germany) analysed in 2004 a total of 37 papaya fruits that were imported into the EU from different origins. Ten out of 13 papayas derived from Hawaii tested positive for the genetic modification, confirming the expected unauthorised import of GM products in a target directed analysis for UGMs. Although the underlying discovery process may be highly complex, the logic behind these cases can be intuitively understood and translated into a structured, systematic and global search action for similar cases. To illustrate this, the proposed search action is broken down in several subsequent conceptualized steps.

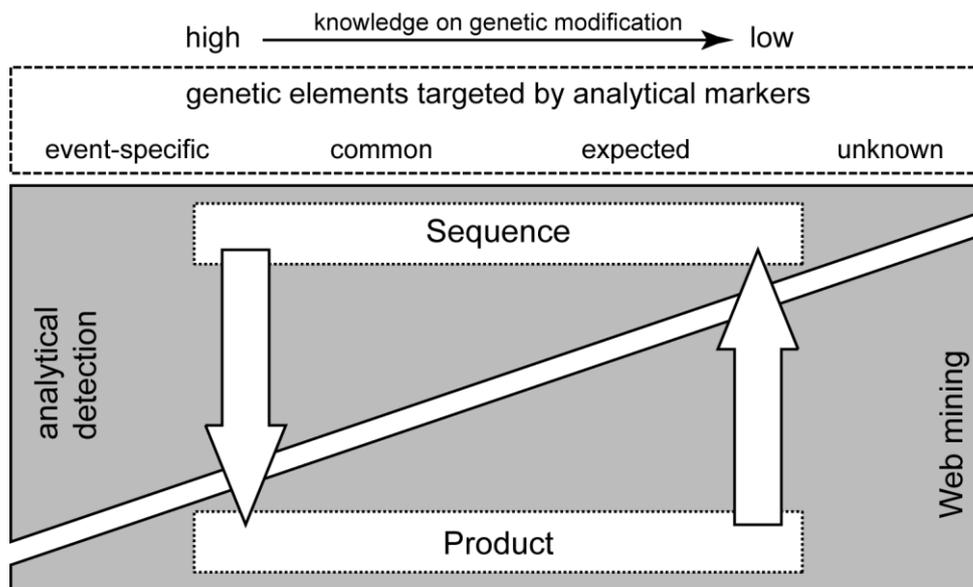


Figure 2 - Two complementary strategies: “sequence-to-product” and “product-to-sequence” are necessary to discover UGMs. The optimal balance between analytical evidence and documented evidence depends on the availability of knowledge required to design an analytical detection method, and the probability and cost to detect UGMs in routine analysis. For products that are not officially registered for marketing, or for UGMs that are “unknown” to authorities, UGM discovery primarily relies on systematic Web-mining to reveal documented evidence for the presence of UGMs on the market, followed by sequence information collection, targeted selection and analytical confirmation of suspected UGMs. (Adapted from Ruttink et al, 2010a)

The proposed discovery process is based on a simple information network (a simple graph structure, representing the current experts’ conceptual model as a “mind map”) that is built around a central named entity “product” (Figure 3), consistent with the notion that, at least in the EU, authorization is granted at the level of a GMO event and products derived thereof, not to sequence markers per se. The conceptual model further describes potential “information resources” and their connections. Using Web crawling software based on a list of descriptive keywords that cover each information resource, the initial information network is refined and extended to cover all possible biotech products. In contrast to analytical detection methods, associative retrieval based on similarity or co-occurrence allows products to be found without prior knowledge of their existence. All the associated information is stored for text mining, filtering and further querying. In a subsequent step, queries are designed that systematically search for “conflicting evidence in documentation” by cross-checking the critical information resources to identify suspicious products.

For example, a query can be designed based on the concept that an advertisement, or import record, for a biotech product without an authorization dossier in the relevant jurisdiction, provides evidence that an unauthorized product is marketed. Or, in a more complicated example, the product description in an authorization dossier can be checked against a patent description underlying a product, or against novel experimental evidence (such as scientific publications, field trial notifications, etc.). Moreover, such a UGM discovery process is typically iterative. As the results of the initial search are mapped to the information network, the network is refined by incorporating unforeseen new sources of data or information. This, in

turn, allows the querying process to be improved. The entire process repeatedly collects evidence to build a case around suspect products, and generates alerts. So far, cases of UGM discovery were the result of the awareness and initiative of individual institutions, and they were often based on routine or targeted inspection of documentation.

UGM discovery may remain incidental, unless experts are better supported in their continuous, highly manual task of searching and linking relevant information. Fortunately, the challenges of knowledge integration in life sciences, which also apply to the UGM discovery process, have been recognized by computer scientists. Web Science initiatives such as the Bison project (<http://www.bisonet.eu/>) have as a target the development of third generation information discovery tools that support the scientific knowledge discovery process and can be employed in the UGM discovery process. Also, the Joint Research Centre of the European Commission has set up a Web Mining and Intelligence real-time media monitoring system (European Media Monitor) scanning thousands of news sources, classifying articles in 30 languages, alerting and informing users about their topics of interest (<http://press.jrc.it/overview.html>). Open Source Intelligence aims to provide a solution to the virtually impossible task to manually process thousands of documents, by developing tools that automate the process, both in the retrieval of documents and in the extraction of information.

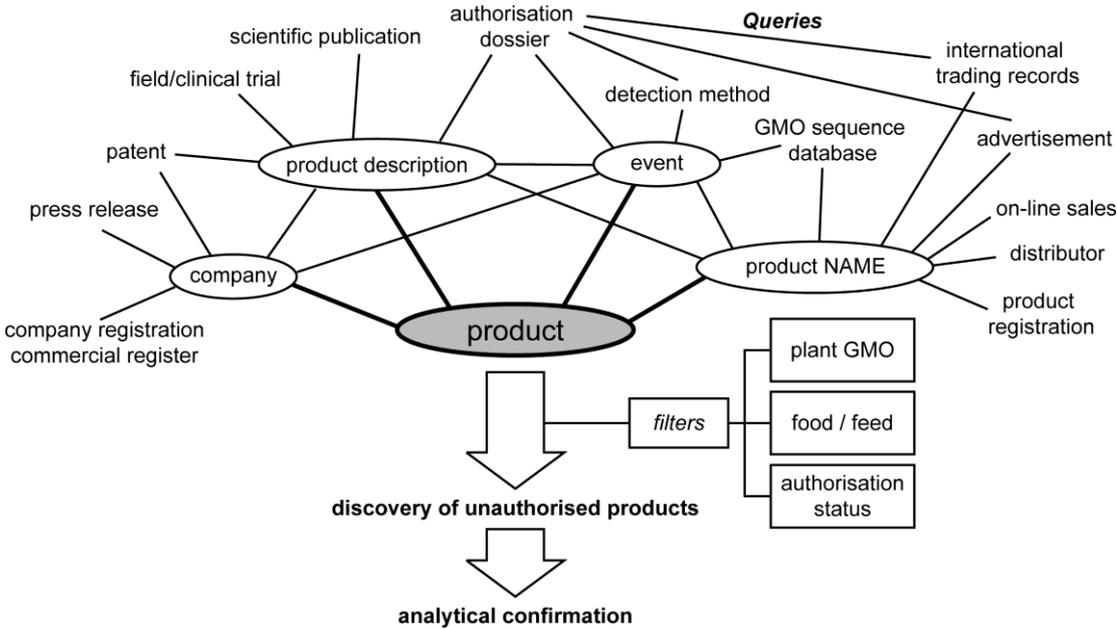


Figure 3 - A mind map built around biotech products functions as initial input to a systematic, structured search for relevant information and guides UGM product discovery.

Similarly, for the detection of authorized GMOs, using information about the product (such as the geographical origin of the products or even the origin of the product ingredients, existence of separated production, processing and transport chains for co-existence) crossed with data about the authorized (synchronously or not) GM events at the origin would help drawing leads regarding the potentially presence of GMOs in the product and eventually what event to look for.

The above proposed approach should therefore help sorting in a cost-effective way the products potentially containing authorized GMO or UGM from the ones being GM-free or containing authorized GM below the legal threshold.

In addition to this approach, using statistics on the presence of GMOs in the tested products may also help orientating further testing. To our knowledge, no harmonized data is available regarding the ratio of the number of GM containing samples vs. tested samples. However, based on data gathered by the National Institute of Biology (Slovenia) from several ENGL members or found on official websites, the percentage of samples found positive (all species and matrix types) for the presence of GMOs oscillates between 10 % and 20 % of the total number of analyzed samples (data from year 2004 to 2011). Taken by crop species, the highest probability to find GM events is in soybean samples (8 % to 15 % of positive samples) followed by maize (2 % to 5 % of positive samples). Focusing on matrix types, the ratios vary greatly between seed (2 % to 5 % of positive samples, mainly in maize), food (8 % to 15 % of positive samples, mainly in soybean and maize) and feed (23 % to 83 % of positive samples, mainly in soybean and maize). Making these types of data available to the competent authorities and the laboratories, including the frequency of each authorized event in the different types of matrices, may help better targeting the screening for authorized GMOs and may therefore significantly increase the cost-efficiency of GMO detection.

5.4 Proposal on how the approach could contribute to the efficiency of the overall GMO detection strategy

At the moment GMO detection occurs mainly via a non-targeted approach. In most of the cases the question sent with the sample is to test for the presence of GMO and, if positive, to quantify the GMO content. The sampling is based on a sampling plan and in most of cases aims to cover the complete food and feed market.

However recently some slight modification has been introduced in the approach, e.g. in the testing for UGM in Chinese rice according to Commission Implementing Decision 2013/287/EU. Both the sampling and the analytical procedure applied on these samples are already partly product centred. This approach could be further developed and structured on the basis of collected information. In practice this means that the question, which is until now in most of the cases “to detect and quantify GMO in a product” shifts to the question “is a particular GMO or a group of GMOs present in a rice product”. By specifying the question sent to the laboratory the efficiency of the analysis will be improved. Ideally this request to the laboratory should be driven by the question the Competent Authority wants to be addressed for the particular sample and by information collected on the sample.

Depending on the goal to be achieved with the analytical testing, different approaches could be developed, i.e. a sampling strategy combined with a dedicated question. This idea could be summarized as a “pro-sampling and -analytical decision tree”:

- Monitoring for correct labelling of authorized events. Here the focus could be on those commodity products that are known to have a high chance to contain an authorized GMO: e.g. at the moment maize and soya. The testing could focus on screening elements plus event specific elements for GMOs of the species to be tested and of which it is known that the event is grown in the area from where the product is originating.

- Testing for botanical impurities and the potential presence of GMOs in the impurities;
- Testing for asynchronously authorized GMO events (low level presence);
- Testing for unauthorized events,

.....

In function of the analytical experimental approach different sets of information could be of interest to improve the efficiency of the testing:

- traceability data (such as origin of the products and the potential cultivation of GMOs in the region, countries involved in the transportation, and data about the transportation of the product)
- composition of the sample
- GM products (traits) under development,
- IP documents on products under development,
- advertisements on products to search for,
- authorization status on GMO,

....

5.5 Practical tools for the estimation of the risk of GMO presence

For qualified experts traceability data that accompany the product and provide information on its geographical origin and path in the production/supply chain, can be used as a source of information (expert knowledge) that, represented in a decision tree, can provide a quick indication for the analysis of a given issue. Software implementation of the model could ask the user for the documentary traceability data and provide qualitative estimation.

Within the CoExtra project ‘GM and non-GM supply chains: their CO-EXistence and TRAcability EU FP6 Integrated project 007158’ two decision support systems (DSS) were developed: the Unapproved GM Model (UGM) and the Transportation Model (TM). The UGM was developed to assess the risk of contamination with UGM varieties based on product traceability data. The TM was developed to assess the chance of commingling of GMO-free batches with authorized GMO varieties by analyzing the production chain from field to food or feed producer (Bohanec *et al.*, 2013). The estimation of this risk is approximate and qualitative and measured as very-high, high, medium, low, or very-low.

As an example the decision tree UGM is shown in Figure 4. Relevant information about the problem is gathered and decisive factors are formalized. This is a simple decision tree that was gradually extended and converted into a hierarchical rule-based model (Figure 5). The model was implemented in DEXi decision modelling toolbox (Bohanec, 2008) and the UGM contamination risk was determined according to four main sub groups of attributes:

1. Geographical origin of the product. In principle, the risk depends on region of production and is generally higher for regions of high GMO production.
2. Systems used in previous stages of the supply chain in order to produce traceability data.

3. Logistic strategies that were used to take the product to the current point in the supply chain. The more complex the logistics, the higher is the likelihood of mixing the product with GMOs of unknown origin.

4. Analytical Methods that might have been applied previously and whose results may be (but are not necessarily) available at present.

The four sub models address the complexity of the attributes by including records of different scenarios and available data (e.g. on subsequent steps in the production chain (e.g. different systems of traceability in place).

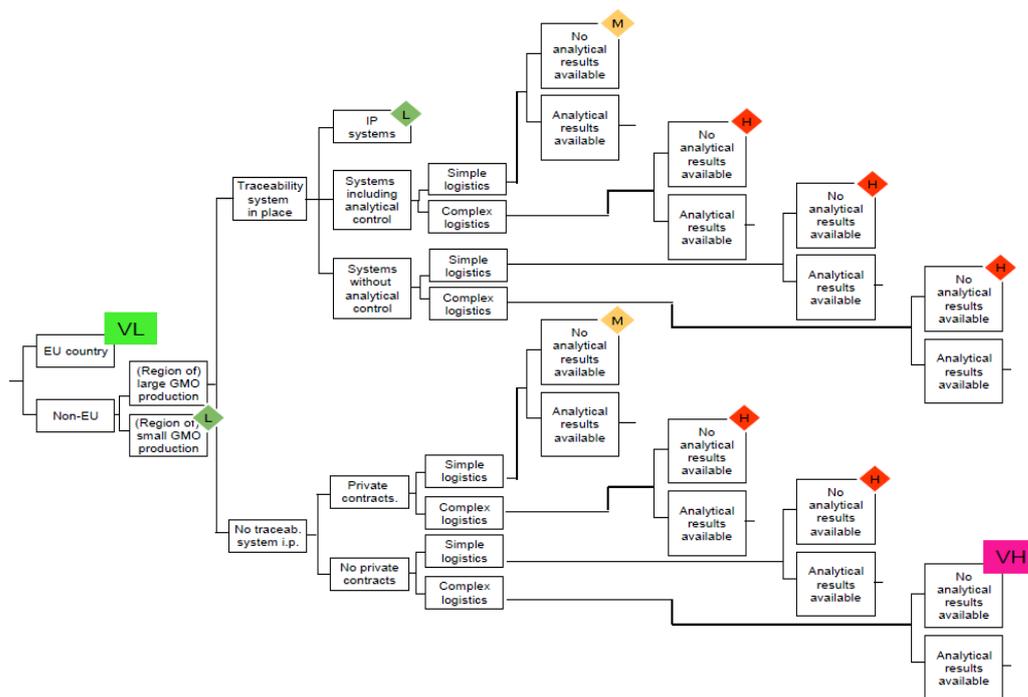


Figure 4 - Decision tree diagram for the assessment of the risk of the presence of UGMs (Žnidaršič *et al.*, 2009).

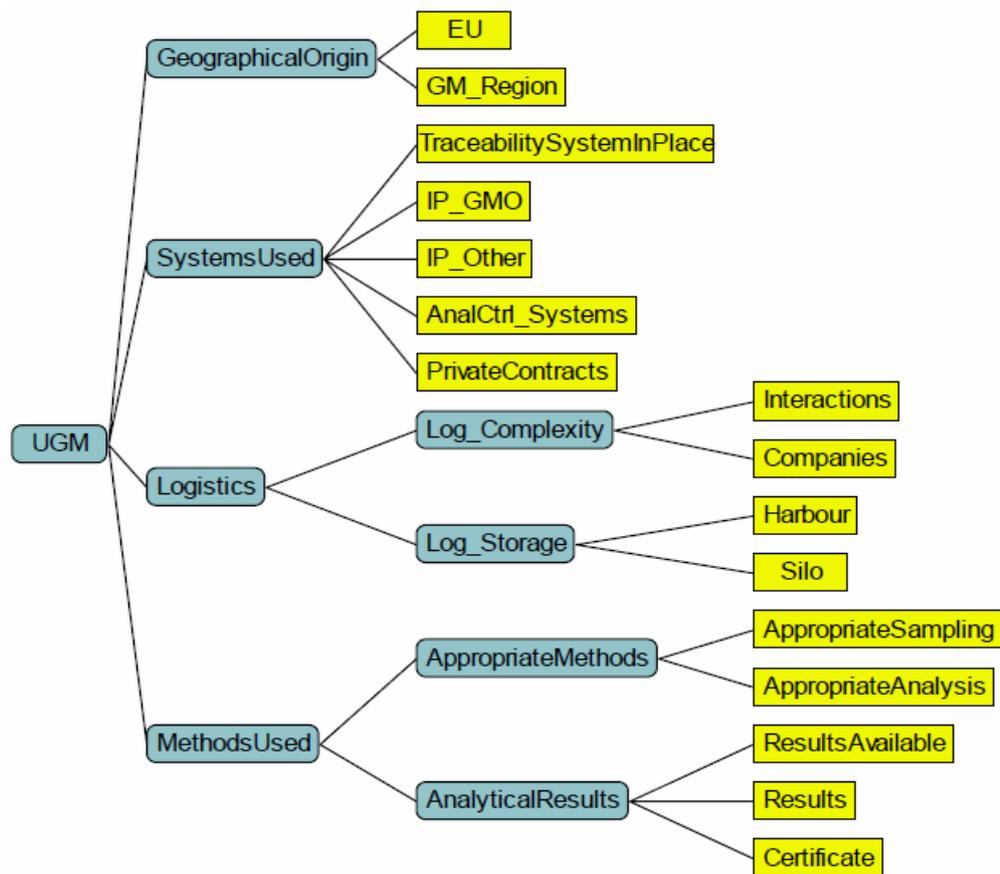


Figure 5 - Structure of the hierarchical rule-based model for the risk assessment of UGMs (Žnidaršič *et al.*, 2009).

The decision tree representation is simplified regarding the contents, but it is also easier to use. It can be implemented as web-application with a user interface in the form of a wizard. On the contrary, the model of DEX methodology needs all data input at the start but it is robust and can handle missing inputs. The hierarchical model is bound to DEXi software, which runs directly only on MS Windows platforms, so, the use of the model is not as simple as in the former case.

The model in decision tree form cannot be easily expanded when new knowledge became available as the tree exponentially ‘explodes’ but this is not an issues in models of DEX methodology. Consequently the hierarchical rule-based models usually describe problems in much more detail. The developed decision tree model was able to cover 27 different situations, whereas the DEX model covered 147,456 different input combinations.

The two DSS have different characteristics, which makes them suitable for different application and makes them complementary. Both of them cover different level of simplicity and completeness and of redundancy and coverage; therefore they represent a useful combination of approaches to a single problem. The UGM model is currently further developed within the frame of the European Decathlon project (<http://www.decathlon-project.eu/>).

Given the increasing complexity of GMO testing, we suggest that testing laboratories move towards a knowledge based testing strategy when possible. The decision trees shown above could be adapted, used and shared between ENGL members through the ENGLnet website.

6. Next Generation Sequencing (NGS) in the GMO testing laboratory

6.1 Advent of Next Generation Sequencing

Since the discovery and characterisation of DNA, scientists were always fascinated by the possibility of deciphering DNA sequences, which is something potentially pertinent to all branches of biology and medicine. With the introduction in the 1970s of Sanger Dideoxy Sequencing (also known as Sanger), scientists began to decode the DNA, the key-code of life for any biological system, and to better interpret the overall scheme of the genetic information. The Sanger technology quickly became well implemented and widely used in laboratories around the world. Moreover, the Sanger sequencing process was largely automated. The most important application of the automated Sanger sequencing was the determination of the complete sequence of the human genomic DNA (International Human Genome Sequencing Consortium, 2001; Venter *et al.*, 2001), achievement of The Human Genome Project, probably the first worldwide/global experiment, fully completed in 2003 after 10 years of work and millions of dollars of investment in research.

The day after the Human Genome Project, it was clear that the idea of large-scale sequencing of other complex genomes was prohibitive and even the re-sequencing of the human one was too complicated and expensive. In fact, even if automated, the capillary electrophoresis sequencing has intrinsic limitations in terms of throughput, scalability and resolution. As a consequence, Sanger sequencing seemed to represent a real bottleneck, preventing scientists from obtaining the essential information they need for better understanding complex biological systems.

To overcome these limits, since the end of the 1990s new sequence approaches have been developed. Within a short period of time between 2004 and 2006, new sequencing methods and machines were quickly set up, based on different chemical principles compared to the classical methods used until then. For this reason, the term Next Generation Sequencing (NGS) was coined (see a review in Metzker, 2010, Hong et al, 2013).

Briefly, the key concept is that NGS is based on the sequencing across millions of reactions in a massively parallel method, rather than being limited to a few DNA fragments, thus producing millions of sequences in one single experiment. Moreover, new NGS technologies are being developed, so that terms like "Second" and "Third" Generation Sequencing technologies are now commonly used (Ozsolak, 2012).

The main advantages of NGS technologies (commercially available since 2005) are both time and cost reduction. With the automated Sanger classic method, the cost for a single sequenced base is of around 0.5 euro. With a NGS sequencer, sequencing the same base costs less than 0.001 euro. Nowadays, with the advent of NGS, hundreds of completed genome sequences from higher organisms (i.e. plants and animals) are available, well annotated and browsable (see for example *The Ensembl project*¹), thousands from

¹ <http://www.ensembl.org>

bacteria and fungi² (Pagani *et al.*, 2012), hundreds re-sequenced human genomes³ (Durbin *et al.*, 2010). Compared to the Human Genome Project, currently, with NGS sequencing machines, it is possible to sequence one's genome in a few days for about 5000 euros, but many companies are claiming that very soon they will offer sequencing of one person's DNA for \$100.

6.2 NGS in the laboratory

With the introduction of NGS technology in the laboratories a major transformation occurred in the way scientists retrieve genetic information from biological systems. Being strictly related to DNA, NGS technology is nowadays considered a revolution in particular in the fields of Life Sciences, actively contributing to the birth of the so-called new emerging *-omics* sciences (i.e. genomics, *transcriptomics*, *epigenomics*, *metagenomics*, *canceromics*).

The impact of NGS is really impressive both in terms of applications and fields: a summary of already used NGS applications in different research fields is shown in Table 2, where it is clear that scientists are using NGS to break down many limits to their comprehension and advancing scientific fields, from human disease to evolutionary science, from agriculture to ecology.

Table 2: NGS applications on different biological fields

Type of application	Biological field						
	Cancer research	Evolution & ecology	Human genetics & genomics	Metagenomics & microbial diversity	Microbes, viruses & infectious diseases	Model & non-model organisms, systems biology	Plants & agricultural biotechnology
Ancient DNA	-	X	X	X	-	X	X
ChIP-seq / Methylation / Epigenetics	X	-	X	-	X	X	X
Eukaryotic Whole Genome Sequencing	X	X	X	X	X	X	X
Expression tags	X	-	X	-	-	X	X
Genetic variation detection	X	X	X	X	X	X	X
HIV sequencing	X	-	X	X	X	X	-
Metagenomics and Microbial Diversity	X	X	X	X	X	X	
Mitochondria / viruses / plastids / plasmids	X	X	X	X	X	X	X
Prokaryotic Whole Genome Sequencing	-	X	-	X	X	X	X
Sequence Capture / Target Region Sequencing	X	X	X	X	X	X	X
Small RNA	X	X	X	X	X	X	-
Somatic variation detection	X	-	X	-	X	X	X
Technology and Informatics	X	X	X	X	X	X	X
Transcriptome Sequencing	X	X	X	X	X	X	X

X means that scientific research article have been published. Adapted from the repository of articles on Roche 454 website, <http://454.com/publications/publications.asp>

6.3 NGS and Bioinformatics

NGS data throughput requires an adequate informatics infrastructure and specific programs for data analyses. Like every other produced sequence, the sequences are usually conserved in collaborative databases. Before the advent of NGS, those databases consisted of billions of bases; now the values are in the trillions. Bioinformatics, the scientific field dedicated to solving biological problems at the molecular

² See for example <http://www.genomesonline.org/>

³ See for example <http://www.1000genomes.org/>

level using informatics tools, provides an important support to these activities, to the point that the two are often named together and it is a common scientific opinion that NGS cannot exist without Bioinformatics (Hong *et al.*, 2013). An adequate informatics infrastructure, depending on the type and scale of the intended project, may include:

- High capability data storage devices, comprising those for back-up purposes, in terms of hundreds Terabytes.
- High performance computing (HPC) devices able to analyse large amount of data, such as computer clusters or servers able to run software in scheduled/parallelised environments.
- Stable and high-speed network connections between the HPC devices and the storage arrays.

Moreover, specific competencies to analyse and interpret the data are required, including not only those in bioinformatics, but also in biostatistics and software development.

6.4 NGS and GMO detection: overview of suitable NGS approaches

According to the evidence previously described, NGS has been increasingly used in the GMO-related research fields. Obviously, development of standardized protocols for GMO detection by NGS is still ongoing and different types of existing NGS approaches can be used, basically according to the specific research needs. In any case, their potential is quite impressive.

An overview of NGS approaches potentially applicable to GMOs is reported in Table 3, together with possible strategies. They correspond to:

- **Targeted re-sequencing:** provides an efficient method to quickly characterize specific genomic regions of interest, from PCR products (called "amplicons") up to 50 Mb. Possible strategies include:
 - *PCR and long-range amplicons*, which is most used application of targeted re-sequencing.
 - *Sequence capturing*, that is an efficient enrichment protocol to isolate large or highly dispersed regions of interest from a pool of DNA molecules.
 - *High-Throughput amplicon sequencing*, a special targeted re-sequencing workflow that allows to sequence more than 2,000 amplicons in one experiment.
- **De novo sequencing:** suitable for decoding new uncharacterized genomes and/or genomic traits. Possible strategies include:
 - *Shotgun sequencing*, i.e. sequencing of genomes never sequenced before.
 - *Paired end sequencing*, that is the possibility to sequence 5' and 3' ends of a DNA fragments of fixed length (up to 20Kb) and then use them as "tags" for reconstructing the genome.
- **Metagenomics:** studies the samples in terms of "genome diversity" by sequencing directly their genetic material; it is usually conducted on environmental samples. Possible strategies include:
 - *Shotgun sequencing*, a random fragment sequencing application that is used on a sample derived from a pool of organisms.
 - *cDNA sequencing*, for detection of (un)expected transcripts
 - *rDNA sequencing*, in order to find organism-specific differences in the sequence of variable regions and thus allow identification of the source organism using e.g. 16S rDNA.
- **Transcriptome sequencing:** analysis of messenger RNAs (mRNAs) that represent the comprehensive transcriptome of an organism. Possible strategies include:

- *cDNA library sequencing*, aimed to look for specific transcripts
- *(Low-Input) RNA sequencing*, that enables whole transcriptome analysis of samples with as little as 500 pg of total RNA.

Table 3: Possible NGS approaches applied in the field of GMOs.

GMO application targets		NGS approaches/strategies										
		Targeted sequencing				De novo sequencing		Metagenomics			Transcriptome sequencing	
		PCR amplicons	Long-PCR amplicons	Sequence capturing	High-Throughput amplicon	Shotgun	Paired End	Shotgun	cDNA	rRNA	cDNA Library	Low-Input RNA Samples
<i>(Re-)Sequencing of</i>	Single GMO events	X	X		X							
	GMO Stacks	X	X		X							
	Mixed samples	X	X	X		X	X	X	X	X	X	X
	New GMO plants			X		X	X	X	X	X	X	X
<i>Development of new methods for</i>	Sample composition	X	X	X		X	X	X	X	X	X	X
	GMO quantification	X	X	X		X	X	X	X	X	X	X
	GMO detection	X	X	X		X	X	X	X	X	X	X

6.5 NGS and GMO application targets

As summarised in Table 3, those approaches can be used for different GMO application targets, like molecular characterization of single/stacked known/unknown GMO events, full sequencing of new GMO plants, evaluation of sample composition with one or more GMOs, but even for the development of new methods to determine the exact sample composition or to detect and quantify GMO at very low levels.

Each possible approach listed above can also be considered as a starting point for further research studies: for example, according to the aim of the analysis, NGS on "Mixed samples" can be approached in different ways, like:

- in mixed flours, by sequencing conserved regions in different species for determining ingredient composition;
- in processed food, by choosing and sequencing specific panels of commonly used transgenic DNA traits for GMO detection and/or quantification in food/samples;
- in fresh food, by choosing and sequencing the whole transcriptomes and then looking for GM-specific transcripts.

Moreover, the transfer of sequencing output into a knowledge based system should be suitable for the application in further strategies, such as routine analysis by sequencing or PCR methods.

The application of NGS strategies to detect and identify GMOs and UGMs in a single assay in complex products is now also one of the research goals within the frame of the European Decathlon project (<http://www.decathlon-project.eu/>).

6.6 Current practicability

Some approaches applied to GMOs are in a quite advanced state: for example, in the case of NGS applied to the molecular characterization of the GM event, i.e. sequence determination, a first approach for the identification of events' junction sequences by NGS has been proposed in 2012 (Kovalic *et al.*, 2012),

followed in 2013 by other papers describing the usage of NGS approaches to fully characterize GM events (Yang *et al.*, 2013; Wahler *et al.*, 2013).

In other cases there are still some difficulties: for example, in the characterization of metagenomes, to our knowledge *metagenomics* is currently applied only to bacteria.

Sequence capturing strategies seem to be a very promising and versatile approach for GMO detection and quantification. Sequence capture arrays allow the specific enrichment of genomic regions from full genomic DNA. This approach is applicable to large genomic regions, whole exomes, or any other targeted regions that should be re-sequenced. The set-up of the array is quite expensive and requires sharing sequence information with private companies, which could be an issue in case of patented sequences or confidential data.

It is important to remark again that, whatever approach is used, the most difficult part is the data analysis and data interpretation, which again passes through bioinformatics. For example, in case of re-sequencing strategies, a comprehensive set of whole genome sequence data and bioinformatics tools for analysis of characteristic differences in the genomes of GMO, as well as of parental cultivars and stacked gene crossbreeds, constitutes a basic requirement. Moreover, in particular cases like *transcriptome sequencing* or *de novo* sequencing of large genomes, the bioinformatics impact plays a relevant role on the progress of the whole process and, if not well conducted, it may result as a limiting step for data interpretation and production of the results.

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