Guidance document from the European Network of GMO Laboratories (ENGL): Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials

Arne Holst-Jensen, Yves Bertheau, Theo Alnutt, Hermann Broll, Marc de Loose, Lutz Grohmann, Christine Henry, Lotte Hougs, William Moens, Dany Morisset, et al.

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ENGL *ad hoc* working group on "unauthorised GMOs"

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European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information
Address: Molecular Biology and Genomics Unit
E-mail: JRC-BGMO@ec.europa.eu
Tel.: +39 0332 789379
Fax: +39 0332 786159

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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Prepared by the

ENGL *ad hoc* working group on “unauthorised GMOs”

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Introduction

The *ad hoc* working group on UGM was established on the basis of a mandate adopted by the ENGL steering committee on January 31st 2007. The working group has been chaired by Arne Holst-Jensen, National Veterinary Institute (NVI), Oslo, Norway, and by Yves Bertheau, Institut National de la Recherche Agronomique (INRA), Versailles, France. The other members of the working group have been: Theo Alnutt, Central Science Laboratory (CSL), UK; Hermann Broll, Federal Institute for Risk Assessment (BfR), Germany; Marc de Loose, Institute for Agricultural and Fisheries Research (ILVO), Belgium; Lutz Grohmann, Federal Office of Consumer Protection and Food Safety (BVL), Germany; Christine Henry, CSL, UK; Lotte Hougs, Danish Plant Directorate (PDir), Denmark; William Moens, JRC, Italy; Dany Morisset, National Institute of Biology (NIB), Slovenia; Jaroslava Ovesna, Research Institute of Crop Production (VURV), Czech Republic; Sven Pecoraro, Bavarian Health and Food Safety Authority (LGL), Germany; Maria Pla, Consejo Superior de Investigaciones Científicas (CSIC), Spain; Theo Prins, RIKILT Institute of Food Safety, Netherlands; Daniel Suter, Swiss Federal Office of Public Health (FOPH), Switzerland; David Zhang, Groupe d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Semences (GIP-GEVES), France and Marc Van den Bulcke, European Commission Joint Research Centre (JRC), Italy.

The mandate of the working group was the following:

1. Undertake a review of the currently used detection methods in relation to their ability to detect unauthorised genetically modified materials (UGM), and their reliability in relation to discrimination of UGM from other GM.
2. Identify any gaps and emerging needs to ensure that the ENGL members can either detect and identify UGM or at least have reliable evidences of the presence of such GMOs.
3. Develop guidance on a harmonised approach for detection, and on interpreting and reporting the results of application of detection strategies for UGM. Reporting could e.g. include updating a database. Guidance may also have to consider rational resource use in relation to the diversity of GM materials that may be found in different products.

NOTE: The issue of UGMs in relation to possible bioterrorism will have to rest outside the mandate of the WG until more research and data on the relevance and possible means to cope with the problem is available (e.g. from the Co-Extra project).

The final version of this report was first presented to the ENGL steering committee on September 26th 2010, adopted unanimously by the ENGL steering committee on February 23rd 2011. It was first published on the internet (http://gmo-crl.jrc.ec.europa.eu/) on 27th September 2011.

This overview document is prepared in the frame of the mandate from the European Network of GMO Laboratories (ENGL), in reference to technical issues derived from the authorisation of GMOs according to Regulation EC 1829/2003 (European Commission, 2003) and Council Directive 2001/18 (European Commission, 2001).

This initiative is further motivated by repeated incidents where genetically modified (GM) materials unauthorised in the European Union have been found by enforcement laboratories and others (see also http://gmo-crl.jrc.ec.europa.eu/).

The aim of this document is to provide laboratories of the ENGL with harmonised strategies and guidelines on how to cope with unauthorised GM materials (UGM), as well as to interpret and report analytical results and related information to the competent authorities.

It is the objective to facilitate detection of UGM, if present, without the requirements for a completely new detection paradigm. Therefore, the document also includes a review of presently available approaches for detection of UGM.

Regulation (EU) No 619/2011 (European Commission, 2011), the so-called "Low Level Presence" regulation, entered into force in July 2011 and specifies particular requirements for the analysis for presence in feeds of particular UGMs for which authorisation is pending or has expired. The present guidelines have been adapted to the requirements of this new regulation.

Executive summary

Unauthorised GMOs (UGM) and derived materials are not uncommon in products found on the European market. At present there is zero tolerance for UGM in the EU. In most documented cases, the UGM concentration relative to the product in which the UGM material is found, was low. Low level presence will always represent a challenge to analytically based detection, in particular if the UGM is obscured by other GM material.

Sampling is related to detectability. However, the present document does not present *ad hoc* sampling recommendations for UGM. Analytical *ad hoc* implementation of the zero tolerance for particular UGMs in feed is described in Regulation (EU) No 619/2011 (European Commission, 2011).

In Europe, GM detection is predominantly achieved with polymerase chain reaction (PCR) derived methods targeting the transgenic construct and insertion site DNA sequences. The increase in number and divergence of GMOs developed and commercialised has gradually forced the GM detection laboratories to rationalise their analytical work, and most laboratories now apply initial PCR based screenings followed by (when appropriate) more specific PCR based identification and quantification.

The detection of any GM is dependent on availability of suitable detection method(s) and control materials to verify the performance of the method(s). Other information, e.g. describing the novel trait, introduced genetic elements, etc. may also facilitate detection, verification and identification of the GM. For UGM, this is a major challenge, and the GMOs are therefore classified into four knowledge groups in this document. This classification may facilitate stakeholder communication and decision making in analytical laboratories.

Here it is recommended to apply the same analytical paradigm to UGM detection and detection of authorised GM. This is believed to be cost effective without resulting in unacceptably low likelihood of detection of UGM. The recommended approach is referred to as the ”Matrix approach”, and is currently implemented by many of the ENGL members. It is acknowledged that some UGMs may not be detectable with this approach, and alternative strategies are therefore briefly discussed. However, none of these alternatives are considered universally applicable but instead represent *ad hoc* alternatives where available information, laboratory capacity, etc. may play key roles.

A decision tree is presented, summarising the recommended principles of GM and UGM detection. Notably, the state-of-the-art of GMO analysis is not static, and it is expected that the guidelines and recommendations presented in this document will have to be modified on a regular basis. Finally, the document highlights a number of R&D priorities and points out the need for reinforced information sharing at the global level.

Definitions

- **LOD_{abs}** = absolute limit of detection.
    - Note: The LOD_{abs} is the lowest nominal (average) number of PFU (see definition below) in the template volume distributed to individual PCRs that would allow for an acceptable probability of detecting the target (see definition below).
- **LOD_{pract}** = practical limit of detection.
    - Note (adapted from ISO 24276 [2006]): The LOD_{pract} is the lowest relative quantity (concentration) of the target DNA that can be detected, given a known (determined/estimated) number of species specific reference gene copies (determined as PFU; see definition below).
- **Matrix** = a relational representation of the correspondence between GMOs and their corresponding species on the one hand and the response to specific tests on the other hand (in the text below commonly indicated as 'GMO matrix').
    - Note: Specific examples of different types of 'GMO matrices' are the so-called 'screening matrix' (applied in the detection of GMO presence typically using tests that target elements common to various GMOs) and 'GMO Reference Matrix' (a matrix representation used as decision support wherein the validity of the relationships is supported by experimental or equivalent data).
    - Note: Must not be confused with a material subject to testing, e.g. a soybean flour
- **PFU** = PCR forming unit. A PCR forming unit correspond to a single unit of a DNA target (see definition of target below) that is amplifiable with polymerase chain reaction.
    - Note: DNA target copies can be aggregated and therefore not randomly or homogeneously distributed in a template DNA solution (e.g. tandem repeated ribosomal RNA genes) or damaged and therefore not amplifiable with PCR. The term PFU is more precisely describing the distribution and performance of the target copies in a template DNA.
- **Test** = analytical experiment performed with the purpose to determine whether the corresponding target is present or absent in the material subject to analysis.
    - Note: The test is performed with a specific testing module, e.g. a PCR module with a specified set of primers and probe, reagent concentrations and cycling profile.
- **Target** = a specific analyte distinct in that it is the only analyte that gives a positive response to a specific test.
    - Note: The target is for example a specific DNA sequence motif defined by a set of terminal primer motifs and an internal probe motif.

1 Background

Genetically modified organisms (GMOs) and derived products have been on the market for more than one decade. The acreage and diversity of GMOs (taxonomically and with respect to the genetic composition of inserted sequences) is constantly increasing (James, 2010) and detection is gradually becoming more and more challenging. A wide range of technologies with various levels of specificity, potential or not for quantitation, multiplexing, speed and cost-efficiency are available, see annexes (chapter 5) for a brief review and e.g. Davison and Bertheau (2007) and Holst-Jensen (2003; 2007; 2009a). Enforcement laboratories must comply with legal requirements, but also have to balance their various needs and technological possibilities against available resources, etc. Consequently, there is a clear need for guidance for all stakeholders to apply harmonised strategies for detection, interpretation and reporting of analytical results in relation to the presence of GMOs in the European Union but also applicable to the European Economic Area (EEA = the European Union, Norway, Iceland, and Lichtenstein) and Switzerland.

Based on legal requirements in the EU, the enforcement laboratories have primarily focused on testing for compliance with labelling requirements, i.e. on detection and quantification of EU authorised GMOs and derived materials. To date, any findings of UGM on the European market are to be reported through the Rapid Alert System for Food and Feed (RASFF; https://webgate.ec.europa.eu/rasff-window/portal). In some cases, such alerts become subject of emergency measures (e.g. European Commission, 2005; 2006b; 2006d; 2008). The response to these rapid alerts and emergency measures has required considerable follow up and (financial) input by the enforcement laboratories. As new UGMs emerge new fit for purpose tools for detection are needed to support enforcement laboratories and competent authorities. This will require revision of current analytical procedures.

The present report is particularly focusing on the food and feed supply chain in the EU, although several parts of the document will have relevance also for other applications and outside the EU. The working group considered that the primary requirements for detecting UGM presence are the availability of information on the origin and nature of the UGM (especially molecular data), of reliable detection methods, and of suitable reference materials. The availability of information on sequences, detection methods, reference

material, etc. for a UGM varies considerably, e.g. depending on where it is developed and whether it is commercialised or not outside the EU. Reliable documentary traceability is very important because it can support competent authorities and other stakeholders in efforts to determine if analytical methods are necessary, and to reach conclusions on the possible presence of UGM.

As the availability of information on analytes is key to GMO detection, it is proposed to differentiate GMOs into several classes according to the information available on the analytical target (usually DNA sequences) present in each GMO (= knowledge-based classification; see section 1.1).

It is proposed that the so-called "matrix-based approach" (see Chapter 2) is the most efficient and cost effective strategy to detect accidental occurrence of UGM, as this approach is equally useful for the general detection of authorised GMOs and does not require a new GMO detection paradigm.

In a strictly EU legislative perspective, a GMO is either authorised or unauthorised (European Commission, 2001; 2003). EC decisions with relevance to defining the status of GMOs are published on an *ad hoc* basis. This is for example done when a GMO is withdrawn from the market (see e.g. European Commission, 2007). The published overview should be consulted in case of doubt concerning the legal status of a particular GMO (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). Regulation (EU) No 619/2011 (European Commission, 2011) describes specific requirements for the analysis for presence in feeds of particular UGMs for which authorisation is pending or has expired. According to article 7 of this regulation, a list of the relevant GM materials shall be published and updated by the Commission. This list is officially published at the bottom of the webpage http://ec.europa.eu/food/dyna/gm_register/index_en.cfm. This list should therefore be consulted in case of doubt.

For the purpose of this document, we have chosen to focus on the ability to detect and identify UGM. The most convenient approximation may be to compare the genetic structure of the UGM in relation to other products of the same type, e.g. a UGM maize against other GM and non-GM maize. A UGM that is very similar (in terms of inserted genetic elements) to an authorised GMO can be detectable with e.g. element screening or construct specific methods (see e.g. Biosafety Clearing-house website http://bch.cbd.int/database/organisms/). However, particular analytical tools can be required for identification (see section 1.1).

There are presently two main types of GMO detection methods in use, those that target the genetic material subject to modification (DNA) and those that target the resulting product (protein). Methods targeting the phenotype (e.g. herbicide tolerance) have rather limited application, but are used, e.g. by the Association of Official Seed Certification Agencies in their official seed certification schemes. Protein based methods are widely used for rapid screening of plant materials from the field or the harvest, but these methods are usually not suitable for detection of GMO in processed products due to the degradation of the target molecules. Methods targeting DNA on the other hand are more time consuming but offer potentially all required levels of specificity and ability to quantify the target. Quantitation is not required for UGM in the EU, except for those UGMs covered by Regulation (EU) No 619/2011 (European Commission, 2011). However, the use of quantitative methods can facilitate detection of UGM (see section 2.5.1.3 on quantitative differential PCR). Within the EU, there is a general consensus in favour of DNA-based GMO detection (European Commission, 2004; European Network of GMO Laboratories, 2007; 2008; Holst-Jensen *et al.*, 2006), among others because of their superior specificity. The authors of this report have consequently chosen to focus primarily on DNA-based methodologies for UGM detection and identification.

In the following we will discuss and/or provide an overview of strategies for selection and application of analytical methods, sampling, data interpretation and reporting.

1.1 GMO classification based on the level of available knowledge concerning the genetic structure

The number of GMOs in which a particular target is present can increase with time if the same target is included in or produced by new genetic constructs. The actual coverage, i.e. the GMOs in which the target is found can also include UGM.

The specificity of a detection method is linked with its target(s). The degree of homology/similarity between the target(s) of a detection method and the corresponding DNA sequence or protein in the UGM is very important. Lower homology/similarity will result in lower probability for detection with the chosen method. A method with low specificity can have a higher probability of detecting the UGM since the UGM target can differ slightly from its equivalent in another GMO. However, in combination, several tests with low specificity for different targets may provide a specific identification through convergence of indicative identifications resulting from the data interpretation. Therefore, the choice of detection

method is a trade-off between the ability to detect, the resources invested, practicability and the ability to identify and confirm the UGM nature of the detected material. GMOs potentially found in the food or feed supply chain at present can be classified, according to the level of available knowledge concerning their genetic structure, into the following categories:

- **GMOs fully characterised (knowledge level 1)**

  Here the GMO is known in detail. This category includes

  - all GMOs authorised in the EU under articles 7 and 19 of Regulation (EC) No 1829/2003 (European Commission, 2003).
  - GMOs previously authorised in the EU, but not re-authorised (see articles 8 and 20 in Regulation (EC) No 1829/2003 (European Commission, 2003)).
  - GMOs for which the European Food Safety Authority (EFSA) and the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) consider a dossier complete (see articles 7 and 19 in Regulation (EC) No 1829/2003 (European Commission, 2003)).
    - § NOTE: In documents older than 2010 the EU-RL GMFF is referred to as the Community Reference Laboratory for GM Food and Feed (CRL GMFF).
  - GMOs covered by Regulation (EU) No 619/2011 (European Commission, 2011).
  - Detailed information in some cases is available e.g. from applications for authorisation that have not been granted, peer review publications or patent documents, or from direct information provided by a notifier or a third country.

- **GMOs transformed with the same genetic constructs that were used in knowledge level 1 GMOs (knowledge level 2)**

  - GMOs transformed with the same plasmid constructs can have different legal status. For example DAS-59122-7 and DAS-59132-8 (E32) maize (http://gmo-crl.jrc.ec.europa.eu/E32update.htm; European Food Safety Authority, 2008) and A2704-12 and A-2704-21, A5547-127 and A5547-35 (LibertyLink) soybeans. In these cases the DNA sequence of the UGM construct is the same as that of a fully characterised GM and this can facilitate detection of the UGM. However, the insertion site and sometimes also rearrangements will result in sequence divergence. A new event specific method or DNA sequencing is therefore needed for the definite identification of the UGM. The sequence variation can also affect the ability to detect using existing screening element or construct specific methods.

- Gene stacking, where only non-stacked parental GM plant cultivars are fully characterised. Gene stacking is a special case, and has been reviewed (Taverniers *et al.*, 2008). If a non-stacked parent of the stacked GMO is fully characterised, then the full sequence of at least one genetic construct/insert is already known. Retransformed GMOs such as Cotton 15985 (CERA, 2010) are also gene stacked GMOs (Taverniers *et al.*, 2008).

- **GMOs transformed with new combinations of genetic elements that include at least one element also found in knowledge level 1 GMOs (knowledge level 3)**
  - GMOs transformed with at least one genetic element found in multiple GMOs, e.g. the Cauliflower mosaic virus (CaMV) *35S* promoter (P-*35S*; hereafter referred to as P-*35S*), the CaMV *35S* terminator (T-*35S*), the *Agrobacterium tumefaciens* nopaline synthase terminator (T-*nos*) and the genes *cp4-epsps*, *cry*IAb, *pat*, *bar*, *barnase*, *barstar* and *npt*II for which a screening method is available and where the sequence of the element corresponds to the sequence found in a GMO classified under knowledge levels 1 or 2. However, it should be considered that several haplotype sequences are bearing the same popular name (e.g. P-*35S*).
  - Until now most if not all GMOs are obtained by plant transformations using plasmid vectors. While the inserts of interest can differ, only a few varieties of plasmid vectors are available. Since parts of those vectors may have been transferred to the GMOs, the ability to detect sequences of the vectors can constitute an opportunity to detect UGM of level 3 (Tengs *et al.*, 2007; 2010).
  - It can be difficult to determine if the detected element(s) stem from UGM or from legal material, e.g. natural non-GM sources or combined presence of more than one authorised GMO. Control methods for detection of natural non-GM sources (donor organisms) are necessary to avoid some false positive results, but such methods and/or corresponding reference material are only exceptionally available (e.g. the BCCM pENGL™ plasmid collection). Two examples are the P-*35S* and the T-*nos*. These two genetic elements have been widely used for the development of GM plants and are common in numerous authorised and unauthorised GMOs. P-*35S* and T-*nos* can also be found in non-GM plants infected with CaMV and plants naturally transfected by the bacterium *Agrobacterium tumefaciens,* respectively. Therefore, control methods for taxon (species) specific detection have been developed to distinguish between GM

plants hosting these elements due to genetic modification and naturally infected non-GM plants.

- **GMOs transformed with only novel genetic elements (knowledge level 4)**
  - The use of only novel elements in a construct will make the GMO undetectable with any of the currently used detection methods except a generic vector test, e.g. (Tengs *et al.*, 2004), and will imply that the GMO is an “unknown” GMO for the analyst.
  - Notably, a genetic element used in a GMO can be perceived and/or listed in documents as a familiar element, e.g. a P-*35S*, but remain undetectable with available detection methods. One cause of this could be newly introduced nucleotide substitutions, truncations, etc. (i.e. a novel haplotype). In this case, a GMO that would otherwise be considered as belonging to knowledge level 3, will fall in this category, because the sequence characteristics of the element(s) are novel.
  - The use of elements in a construct that are derived from within the species’ genepool (often referred to as cis-genes, auto-transgenes or intra-genes) may also result in failure to discriminate a novel GMO from a non-GM comparator.

2 From GMO detection to UGM detection

2.1 Sampling issues

Sampling for UGM detection shall follow the sampling schemes and recommendations applicable to GMO analysis in general, except when the competent authority provides a mandate for application of another sampling scheme. In the latter case, the competent authority shall indicate how the alternative sampling shall be done. Examples of *ad-hoc* sampling schemes for UGM are found in emergency measures published by the European Commission (European Commission, 2005; 2006a; 2006b; 2006c; 2008). Regulation (EU) No 619/2011 (European Commission, 2011) specifies the sampling to test for particular UGMs in feeds.

The relative limit of detection (LOD), as well as the probability of detecting GMOs including UGM is linked with the sample size. Increasing the sample size will lower the LOD and increase the probability of detection of any GMO including UGM in the product that is sampled. However, increased sample size will also increase costs and different sampling schemes and sample sizes can result in a lack of harmonisation between laboratories in the same or different EU member states as well as between laboratories in and outside the EU.

2.2 The matrix approach as a general strategy in GMO detection

The detection of GMO is performed by targeting either phenotypic characteristics such as production of a specific protein or nucleotide sequences representing the genetic modification. Such targets can be present in many different GMOs, e.g. the common CryIAb protein or the P-*35S* DNA sequence (see "knowledge level 1 to 3"), or be specific to a single GMO, e.g. the event-specific DNA junctions (see "knowledge level 1"). Efficient and cost-effective GMO detection approaches generally apply in the first analytical steps; typically these are tests detecting one or more common targets to demonstrate the (possible) presence of GMO. This approach is generally known as “screening”. However, GMO detection laboratories in their screening analyses sometimes observe positive signals that are not perfectly explicable based on the presence of (only) authorised GMOs.

A major objective with this document is to help detection laboratories, in particular enforcement laboratories, in their efforts to find the explanation for such signals in terms of possible presence of "UGM at knowledge levels 1 – 3". Indeed, any GMO (knowledge levels

1 – 3) can by definition be described on the basis either of its event-specific target motifs, i.e. DNA sequences (knowledge level 1) or on the basis of a combination of other specific targets, including plasmid inserted sequences (knowledge level 1 to 3). The combined properties form the basis for developing GMO detection strategies based on the so-called matrix approach (see below). With the increasing complexity of GM crops used in commerce, it is the opinion of the working group that the matrix approach has the strongest potential for ensuring cost-effective and reliable GMO analysis in the foreseeable future.

Before starting any matrix approach based GMO analysis, it is important first to decide which kind of detection methods will be used (e.g. DNA, protein or other phenotypic trait-based methods). To date in the EU, GMO screening for enforcement purposes is mainly performed with methods for detection of DNA sequences and applying PCR technology. This document is therefore focusing on PCR-based approaches. Some alternative approaches based on other (DNA or protein) methods are documented or referenced in the annexes (Chapter 5).

Secondly, a relation matrix ("GMO matrix") must be established. The word "matrix" is used here in the mathematical sense of the word and must not to be confused with the material subject to analysis. This matrix shows the expected response by individual GMOs to specific tests. If the data tabulated is based on experimental evidence with suitable reference materials then the matrix can be referred to as a "GMO Reference Matrix". If the data is based at least partially on theoretical evidence then the matrix can be referred to as a "GMO matrix". Notably, also verified sequence information, e.g. from dossiers can be used to establish the matrix. This matrix is the basis for the term "matrix approach".

2.3 The general principle of matrix approach based GMO detection

First, a series of (PCR) tests is 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:

- which GMOs that are not detected (for these GMOs one or more elements/analytes is not detected), at the practical LOD (LOD_{pract})
- which GMO that can be present (all elements are detectable), and
- which additional PCR test(s) that could be used for further discrimination among the putatively remaining GMOs.

Many detection laboratories have already for several years started their analyses with screening for e.g. P-*35S* and T-*nos* (the two elements that have hitherto been most commonly present in GMOs). This initial screening can be considered as applying a simple matrix approach where the relation matrix contains only two tests and multiple GMOs.

Provided that the analysis is unbiased, it is possible to conduct the matrix approach based analysis both as a combination of individual single target (single-plex) and/or multi-target (multiplex) reactions. Additional reactions can be performed on the sample when the first results are available, to increase the amount of information for interpretation of the results.

In practice, it can be useful prior to setting up the (PCR) tests to consider e.g. the product type (food, feed or seeds) or other available information to make the analysis as cost effective and potentially informative as possible. Identification of the species from which DNA is present is normally extremely useful in order to understand the possible sources of positive responses to specific tests by the product subject to testing. If this information is not available, it can be appropriate to include species identification tests in the analytical setup. Thus, the set of tests and corresponding matrixes can sometimes best be defined on a case-by-case basis. A common source of unexpected observations is non-declared botanical impurities such as soybean derived material in a “pure” maize ingredient.

2.3.1 Establishing a "GMO matrix":

Analytical method (module) GMO event	ScreenMeth A	ScreenMeth B	ScreenMeth C	ScreenMeth D	ScreenMeth E
GM_one	+	+	+	-	-
GM_two	+	+	-	-	-
GM_three	+	+	-	+	-
GM_four	+	-	-	+	-
GM_five	-	+	+	-	-
GM_six	-	-	-	-	+
GM_seven	+	+	-	-	+

Figure 1. Example of a “GMO screening matrix”, where ‘+’ denotes 'target detected' and ‘-’ denotes 'target not detected' in the different GMOs, based on experimental evidence. Preferably, information on the experiments performed to produce the evidence should also be available, e.g. reference to the method, specification of the reference material that was used to verify the relationship and the name of the laboratory that performed the experiment, etc. The example shown lists only screening methods (“ScreenMeth” A-E) but the matrix could also have included more specific methods (see details in annex 5.2 and 5.3).

A "GMO matrix" is a table where each row represents a specific GMO (authorised and/or unauthorised) while the columns represent the analytical test methods, or vice versa (see Figure 1). The response to the test methods by the specific GMOs are indicated by symbols that will allow the analyst to determine if he/she should expect a positive or negative response for a particular combination of GMO and test. It is very important that the relationship indicated in the matrix is established experimentally whenever possible. The authors of the present document therefore recommend that each relationship presented in the matrix express the (experimentally verified) relationship between a specific GMO and a specific analytical test method. Reference to the detection method, the reference material used and the laboratory responsible for the experimental verification of the particular relationship should be given. In this case a “GMO Reference Matrix” is obtained.

2.3.2 Choice of the appropriate combination of targets and tests for the GMOs and products under analysis:

The optimal combination of tests for GMO presence should cover all targets at knowledge levels 1 to 3, because this will allow for a full discrimination between observations representing authorised and unauthorised GMOs. Depending on the product, the specific situation and the purpose, the number of tests can be more restricted. For example, when screening for absence of GMO, the most cost-effective way is to test for the most frequently occurring GMO analytes (e.g. P-*35S* and T-*nos*) with methods experimentally verified with the largest set of GMOs. In this way, the largest set of GMOs can be detected with a minimum of effort/cost. A table with frequencies of occurrence of screening or construct-specific targets can be very useful, e.g. to identify what kind of screening modules it is useful to develop or implement in a laboratory. However, the optimal solution is to identify how to obtain a maximum of information with a minimum of testing, and this is one of the ideas for example behind the GMOTRACK approach (section 2.5.3.1). The positive response rate observed in the “GMO matrix” itself (see figure 1 above) can be the best starting point for the analytical laboratory. However, if samples are known to contain GMO (e.g. many feed samples), it can be more useful to apply tests that discriminate between GMOs, i.e. to detect targets that are present in only a limited subset of GMO (see as an example the COSYPS system in section 2.5.1.2). Alternatively, a combination of both generic and specific assays can be made by sequential PCR analysis wherein the outcome of the tests guides towards what further tests need to be performed (see as an example the practical approach described by Waiblinger *et al.* (2010), section 2.5.1.1). This would have clear similarities to a decision tree approach. Nevertheless, any matrix approach for GMO analysis will require that accurate relations between methods and targets have been established. For this a

Decision Support System (DSS) containing all relevant information may be required. Such a DSS should facilitate the selection of the optimal matrix on a case by case basis. An example is the GMOTRACK software (see section 2.5.3.1 and http://kt.ijs.si/software/gmotrack/).

2.3.3 Description of the "ideal" matrix approach situation

From the above, it is clear that the matrix approach in the case of GMO analysis covers a very diverse spectrum of specific tests. It is also evident that the optimal combination depends on the product(s) to be tested and may change with time. As the number and diversity of possible GMOs in products is constantly increasing, the content of a GMO matrix supporting the GMO analysis will have to be updated continuously by including new GMOs and eventually new tests (for new targets). Below, a set of (minimal) requirements is listed for establishing an "ideal" GMO matrix adapted to real-life situations for GMO analysis in the EU.

Given that the set of GMOs under analysis is evolving, a so-called "GMO Reference Matrix" should be available which includes tests sufficient to detect and potentially discriminate all the authorised (and unauthorised) GMOs (knowledge level 1 to 3). Modularity is necessary to allow for adaptation to the requirements of the different products. Having a common "GMO Reference Matrix" would enhance transparency and harmonisation of the GMO screening approach in the EU. The ENGL could greatly facilitate the implementation of such a concept and provide valuable feedback as to the practicability of this matrix. Finally, the “GMO Reference Matrix” may be given a formal status as the point of reference that all the ENGL members should refer to when a testing scheme is selected.

The choice between selecting the set of tests on a case-by-case and a generalised basis will probably depend on the situation. If a large number of similar products are tested, a case-by-case set of tests can be fit-for-purpose and cost efficient. If a diverse set of products are tested, a generalised set of tests is probably more appropriate.

The establishment of a "GMO Reference Matrix" requires (preferably certified) reference materials and validation of all tests included, at least for authorised GMOs. Certified sources of genomic DNA (where two or more targets may co-exist in an arrangement unique to a particular GM construct or event) and/or plasmids (where individual targets have been cloned) should then also be available to all laboratories performing the (enforcement) GMO analysis.

Software for optimal selection of combinations of tests should be (further) developed. Such software is to be linked to a GMO database, including all the tests covered in the "GMO Reference Matrix", with links to the documentation on the validation status for the tests. Several GMO databases are (being) developed already but may have to be further developed, integrated and modified/adapted to fit such an application.

In addition, the matrix approach would be greatly facilitated by having a uniform analytical DSS. An example could be the Excel sheets developed for COSYPS (Van den Bulcke *et al.*, 2010, see also section 2.5.1.2), that could be made available to all stakeholders, e.g. through a (dynamic) software application.

2.3.4 Results interpretation applying the matrix approach in GMO screening

After testing a sample for the presence of a defined number of targets, the results are compared with the information in the GMO matrix.

A match between the results and the pattern predicted by the GMO matrix, indicates that material from a particular GMO can be present in the sample. For each specific test, the result is scored according to pre-specified decision criteria. Such criteria are for example defined in ISO 24276 (International Organization for Standardization, 2006). In order to conclude that a particular GMO can be present in the sample, the following requirements must be met:

- The analytical results shall be positive for all tests that are applied and that according to the GMO matrix are predicted to yield a positive signal with material of the GMO in question;
- The number of tests applied shall be sufficient to allow for some degree of discrimination between GMOs that can be present and GMOs that can not be present, based on the observed results.

2.4 The matrix approach as tool for assessing UGM presence (knowledge level 1-3)

The matrix approach as described above aims at covering a maximum set of GMOs. Any positive test result that does not fit into a pattern generated by authorised events only, indicates the presence of UGM in the sample (by definition of knowledge level 1 to 3). Screening results predicting the presence of UGM should preferably be verified by use of

construct-specific or event-specific methods, as well as by donor specific control methods (see annex 5.2.3). If such methods are not available, the interpretation of such results will in most cases require more extended evaluation/analysis applying other technical approaches such as differential semi-quantitative analysis, anchor-PCR studies, DNA sequencing, etc. A more detailed description and examples of these technologies is presented in the annexes (chapter 5). A negative test result, however, will not rule out the presence of a UGM.

2.5 Available tools for GMO analysis in a matrix approach context (knowledge level 1-3)

The matrix approach has been developed already in various formats for GMO screening in a wide range of products (seeds, grains, raw materials, composite food and feed). Below, a number of tools that have been applied on real-life samples and have passed different levels of validation and accreditation are briefly described.

The working group acknowledges that there is a need for method harmonisation among the ENGL members. However, the working group has concluded that there is presently no single assay or tool that appears indisputably superior. On the contrary, several assays and tools, discussed in brief below, appear to have advantages and drawbacks that make each of them attractive in some situations and less fit in other situations. Consequently, the working group recommends a continued discussion within ENGL on the premises of method harmonisation, and primarily wish to endorse the implementation of the matrix approach. For future decisions on method harmonisation, the ability of a screening assay to cover as many GMOs (authorised and unauthorised) with the greatest discriminatory power, cost-efficiently, while at the same time allowing for modifications (e.g. addition of new targets), will be particularly important. The efficiency with which an assay can be updated with validated modules may be critical. Efforts to perform such validations may have to be coordinated by the EU-RL, and should be guided by the ENGL.

Several databases covering different features of GMOs are publicly available while others have only restricted access. Some of the most interesting publicly available databases are listed below, and examples of restricted access GMO databases are also presented.

Finally, some tools that can help designing a suitable GMO matrix (GMOTRACK) or provide guidance on GMO analysis (Co-Extra DSS) are also described briefly.

2.5.1 Examples of GMO analysis tools based on the matrix approach

2.5.1.1 Screening for GMO based on the combination of generic and construct-specific markers

The working groups of German GMO laboratories have developed a screening approach that is based on the combination of generic and construct-specific real-time PCR screening tests (SLMB, 2000; Reiting *et al.*, 2007; LAG, 2007; Waiblinger *et al.*, 2007; Grohmann *et al.*, 2009) and an Excel spreadsheet representing a GMO matrix for evaluation and interpretation of the test results (Waiblinger *et al.*, 2010). At present, the spreadsheet covers a set of five test method targets (P-*35S*, T-*nos*, *bar*, *ctp2-cp4epsps* and P-*35S-pat*) present in approx. 100 different GMOs belonging to 10 food-related crops (see http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/screening_tabelle_gvoNachweis.xls?__blob=publicationFile). The basic information about the presence of the targets is either extracted from databases (Bruderer and Leitner, 2003; CERA, 2010) or by alignments with GenBank sequence entries or other sequence data (e.g. applications for authorisations). All commercially available reference materials containing the targets have been used to experimentally establish the relationship between test method and GMO (represented by the reference material). Thus, for GMOs where reference material is not (yet) available the information in the GMO matrix (i.e. the spreadsheet) is based on theoretical evidence. The use of symbols in the spreadsheet allows the user to discriminate clearly between experimental and theoretical evidence.

Screening is either done step-by-step or simultaneously and is combined with plant species-specific methods in order to determine the species composition of the samples. On the basis of the experimental results, the integrated Excel-based filtering functions of the spreadsheet can be used to condense the number of candidate GMOs present in the sample. In the next step in the analytical process confirmation tests for the candidate GMOs normally apply event-specific methods.

Work is in progress to expand the number of tests and GMOs covered, and to constantly update and broaden the underlying molecular data and experimental evidence.

The approach and tools have been successfully applied by the German network of enforcement laboratories in their routine GMO analyses for food and feed products.

2.5.1.2 COSYPS

CoSYPS stands for "Combinatory SYBRGreen qPCR Screening" and applies a limited set of real-time PCR methods (tests) allowing the user to test for the possible presence of GM events authorised for commercial purposes in the EU (Van den Bulcke *et al.*, 2010).

SYBR®GREEN real-time PCR methods have been developed that target four different types of DNA elements: 1) a generic plant-DNA denominator (plastid *rbc*L isolated from a.o. cotton, rape seed and maize), 2) species-specific elements (soy, maize, oilseed rape, cotton, sugarbeet and rice), 3) generic recombinant DNA elements (P-*35S* from CaMV, T-*nos* from *Agrobacterium*), and 4) recombinant trait-specific elements (*cp4-epsps*, *cry*IAb, *pat* and *bar*). The corresponding amplicons have been cloned into a pUC18 vector and all plasmid vectors can be used in a plasmid-mix set-up as reference material.

In parallel, a mathematical model has been developed that allows for identification of possibly present GM events in a sample applying a prime-number based GMO identification algorithm (Van den Bulcke *et al.*, 2010) The model is developed in a Microsoft Excel format and has been fully operational within an ISO 17025 evaluated system since September 2006.

By July 2010 this GMO detection tool had been successfully applied in about one hundred GEMMA "Food Ingredient" proficiency tests or ISTA "Seed" proficiency tests and in more than 350 different Food/Feed samples by the Belgian GMO enforcement framework under control of the Belgian Federal Agency for Food Safety.

2.5.1.3 Differential quantitative PCR (dQ-PCR) evaluation for UGM assessment

When quantitative PCR modules are applied for screening and identification of GMOs on the same sample then discrepancies between the screening and identification results can be estimated and provide a possible means of detecting UGM (differential quantitative PCR = dQ-PCR; Cankar *et al.*, 2008). For this purpose a variable “µ” is defined as the difference in quantity between a screening element (e.g. P-*35S*) and the identified GMO(s) and natural source of the screening element (e.g. the CaMV).

$$\mu = Q_{screening\ element} - \left(Q_{GMO} + Q_{natural\ source\ of\ screening\ element}\right)$$

Under optimal conditions, µ should equal zero. When µ significantly exceeds zero, this can be interpreted as an indication of the presence of unidentified GMO that can be UGM. In order to apply this approach and conclude that observed deviations are significant, the copy number in the GMO and donor organisms of the different targets need to be experimentally determined prior to the test (the dQ-PCR can also be used for determining these copy numbers in previous experiments). The measurement uncertainty at the Ct-level for each of the targets also needs to be experimentally determined by using replicates.

A dQ-PCR approach could be a valid asset in UGM assessment and could be used to detect UGM presence at any level, depending on the acceptance of the difference value by the analyst, and the power of the test. The combined measurement uncertainty and/or

confidence interval (e.g. 95 or 99%) used by the analyst will have to be included in the test report.

2.5.1.4 "Pre-spotted" plates for event-specific screening

The Molecular Biology and Genomics Unit at the Institute of Health and Consumer Protection of the EC Joint Research Centre (Ispra, Italy) has pursued to develop ready-to-use "pre-spotted" 96-well format plates covering 48 quantitative real-time PCR methods representing GMOs or plant species applicable in common quantitative PCR platforms. Those plates contain all necessary reagents to screen for the presence of the EU-authorised GMOs and a number of unauthorised GMOs (such as Bt10 maize and LL601 and Bt63 rice). These plates were distributed to the ENGL for "proof of concept" testing (Querci *et al.*, 2009) and are currently subject to optimisation for specific customer-directed applications (e.g. single ingredient "pre-spotted" GMO screening plates). A similar approach has been developed, published and tested by the Japanese GMO laboratories (Mano *et al.*, 2009). These plates offer a highly efficient, time-saving, low cost asset in GMO analysis for enforcement purposes. Furthermore methods validated by the EU-RL/ENGL (or others) can be easily implemented in such an analysis format. The requirements set by ISO and *Codex Alimentarius* guidelines to estimate GMO content applying at least 2 sub-samples and to include a positive (and preferentially also a negative control) in all analyses, can in the future represent a major practical bottleneck when continuing with a 96-well format approach. Compared to approaches applying multiplex reactions, the “pre-spotted” plates may also require significantly more template DNA, thereby increasing the DNA-extraction costs. The ability to use this approach to detect UGM is also highly dependent on the targets covered. The pre-spotted plates distributed to ENGL in 2009 did not include screening element methods, and only included methods for a few UGM.

2.5.1.5 Multiplex quantitative PCR screening approaches

Application of high throughput/multiplex detection methods can prove very useful and can apply in the matrix approach. Several methods have been described, but only few of these may be fit as screening tools for UGM detection. These high throughput methods are either based on combinations of one or several oligoplex PCRs followed by multiplex (pooled) identification of the amplified DNA (Hamels *et al.*, 2009; Heide *et al.*, 2008a; 2008b; Leimanis *et al.*, 2008; Nadal *et al.*, 2006; 2009) or apply multiple simultaneous PCRs (Chaouachi *et al.*, 2008; Mano *et al.*, 2009; Querci *et al.*, 2009).

2.5.1.6 Chip based detection methods

A low density micro-array, initially developed during the EC funded GMOChips European research project (http://www.bats.ch/gmochips/), is to date (September 2010) the only multiplex matrix based screening approach that has been collaborative trial validated (Leimanis *et al.*, 2008). This so-called DualChip® combines screening results obtained with generic, construct-specific, trait-specific and taxon-specific PCR methods and is accompanied by software that provides support in data handling, interpretation and reporting. It presently covers mainly EU-authorised GMOs but is amendable to broader platforms. Drawbacks to this technology are i) a lower flexibility with respect to inclusion of novel targets on an *ad-hoc* basis, ii) the need to purchase (relatively) expensive commercial reagents and novel equipment in addition to the PCR apparatus, and iii) the increased risk of carry over contamination resulting from the dependence on post-PCR pipetting of amplified DNA.

2.5.2 Database information for GMO analysis

The following is a list of databases available to at least some ENGL members. Other databases may exist or be developed in the future.

2.5.2.1 Publicly available information

The following databases are examples of publicly available databases with few or no restrictions on information access:

- European Commission Joint Research Centre (http://gmo-crl.jrc.ec.europa.eu/gmomethods/)

This EU Database of Reference Methods for GMO Analysis has been developed jointly by the Joint Research Centre as European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL). It aims at providing a list of reference methods for GMO analysis that have been either validated in a collaborative trial according to the principles and requirements of ISO 5725 and/or IUPAC protocol or verified by the EU-RL GMFF in the context of compliance with an EU legislative act.

- United Nations Cartagena Protocol Biosafety Clearing House (http://bch.cbd.int/)

The Biosafety Clearing-House (BCH) is a mechanism set up by the Cartagena Protocol on Biosafety to facilitate the exchange of information on Living Modified Organisms (LMOs) and assist the Parties to better comply with their obligations under the Protocol. Global access to a variety of scientific, technical, environmental, legal and capacity building information is provided in all 6 of the UN languages.

- Aphis (http://www.aphis.usda.gov/biotechnology/status.shtml)

This database provides information about the status of applications and environmental releases and field tests in the USA.

- Food standard Australian and New Zealand Database (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/gmorec-index-1)

This database contains information on all GMO dealings approved by, or notified to, the Gene Technology Regulator (the Regulator) or specified in an Emergency Dealing Determination. It also contains information on all GM product approvals notified to the Regulator by other regulatory authorities.

- OECD Biotrack (http://www.oecd.org/department/0,3355,en_2649_34385_1_1_1_1_1,00.html

This database includes various consensus/ guidance documents, product database, regulatory contacts of member countries, and field trials database.

- GMO Compass (http://www.gmo-compass.org/eng/home/)

This database among others provides an overview of the current status of all GMOs that have been approved or is awaiting approval in the EU. No molecular data or detection method is provided.

- GM Crop Database (former Agbios http://cera-gmc.org/index.php?action=gm_crop_database)

This database provides among others an overview of the elements inserted into GMOs authorised in one or more countries around the world. No molecular data or detection method is provided.

- GMDD (http://gmdd.shgmo.org/)

This database provides among others an overview of available detection methods, their validation status and to some extent includes molecular data.

- BATS (http://www.bats.ch/gmo-watch/)

The original online database is no longer available. However, a comprehensive printed review report from the Swiss Biosafety Assessment, Technology and Sustainability (BATS), updated last in 2003, entitled "Genetically modified crops: molecular and regulatory details", can be downloaded from this website.

- Patent Databases (numerous databases searchable on the web).
  - http://www.espacenet.com
  - http://patft.uspto.gov/
  - http://www.google.com/patents
  - http://www.wipo.int/pctdb/en/ for international patent search (WIPO)

2.5.2.2 Restricted Access information

- Central Core Sequence Information System (CCSIS) is a database system developed by the EU-RL GMFF that contains detailed molecular data for all GMOs authorised in the EU as well as for several other GMOs. The database can be used in support of enforcement analyses, but presently only via the EU-RL GMFF.
- The EFSA Extranet (https://sciencenet.efsa.europa.eu/portal/server.pt/) is a database managed by the European Food Safety Authority and contains all documentation included in the dossiers submitted to the European Competent authorities for GM product authorisation.

2.5.3 Tools for optimised matrix design and data interpretation

2.5.3.1 GMOTRACK (Novak *et al.*, 2009)

- Generator of cost-effective GMO testing strategies

GMOtrack is a command line utility that implements the GMOtrack algorithm. It generates cost-effective testing strategies for traceability of GMOs. Given a table of GMOs (along with the probabilities of their presence, the genetic elements present in their genome and a linear cost function) GMOtrack computes the optimal set of screening assays for a two-phase testing strategy. The system is able to constantly adapt the strategy to the current situation on the GMO market by updates of the GMO

tables. Additionally, it will enable automatic interpretation of experimental results. This approach lowers the cost and the time needed for each individual analysis thus simplifying GMO testing. The software is downloadable from http://kt.ijs.si/software/GMOtrack/.

2.5.3.2 Co-Extra DSS

- Decision support system

This is a modular software system, which has been developed through a model-driven and data-driven methodological approach, provides several qualitative multi-attribute models that assess various aspects of a.o. analytical methods, sampling plans, and food and feed products. These models capture and represent expert knowledge in the form of hierarchically structured variables and decision rules. The DSS contains a database of methods, products and operational taxonomy units, which is implemented on a server and accessible through a web-based user interface. The DSS functionality is targeted to a variety of potential users: policy makers, farmers, importers, transporters, feed/food producers, retailers, consumers, analytical laboratories, users of test reports from analytical laboratories, and operators and managers of official control. More information can be found on the Co-Extra website http://www.coextra.eu.

2.5.3.3 Unapproved GMO checker

- This is a software developed for application with a specific detection assay developed and applied in Japan (Mano *et al.*, 2009)

The software is downloadable from http://cse.naro.affrc.go.jp/jmano/index.html

2.6 General aspects of GMO detection at knowledge levels 3 and 4

Ruttink *et al.* (2010b) distinguished between analyte centered and product centered detection approaches. The former is typically applied in routine analyses and outlined in the previous sections of this document. In this approach, few assumptions are made *a priori* and the observed pattern of detected and non-detected targets form the basis for interpretation and further analytical work. The product centered approach is more complicated and starts with the analysis of available information leading to the design of an *ad hoc* sampling and detection strategy. In this approach the information analysis (primarily document and computer based) leads to the accumulation of indicia of possible presence of a particular GMO in a particular market niche. *Ad hoc* sampling and detection is then targeted more directly towards the specific market niche and GMO. The product centered approach was

successfully applied to detect and identify a UGM of knowledge level 3 by Ruttink *et al.* (2010b).

The detection of UGM at knowledge level 4 is not easily feasible in a routine laboratory due to the inherent low level of information on the molecular characteristics of this type of materials (see definition in chapter 1) and the kind of detection methods to be used. For this kind of UGM highly sophisticated and often costly technologies are generally required. These testing strategies are therefore considered to fall out of the scope of current routine analyses in enforcement, and therefore described only in brief in the present document (in the annexes, chapter 5).

By definition the analytical laboratory has no *a priori* knowledge about either of the new introduced genetic elements or their associated products or features for a GMO in knowledge class 4 (unknown GMO). In principle, any genetic element can have been introduced. Thus, the obvious starting point would be to compare the suspected GMO with a non-GM comparator and to try to identify differences. Various –omics tools (pyrosequencing, gene expression microarrays, 2D-protein gel electrophoresis, mass spectrometry, etc.) can be applied, but this will only exceptionally lead to detection. Combining the biochemical analyses with well planned bioinformatics strategies to data analysis can increase the success rate considerably. An important question, however, is whether a laboratory will ever receive a sample for knowledge class 4 analysis without at least some additional information, e.g. a brief description of the possible genetic modification. This description can help the analytical laboratory to design a significantly more efficient strategy than a direct blind –omics based comparison of data. A decision support system and other approaches exploiting multiple sources of information treated systematically can also prove useful (Ruttink *et al.* 2010b).

For example, if the sample was taken on the basis of observations of a particular environmental or health effect (phenotypic properties), then there is already some information pointing in the direction of particular classes of genes or proteins. A particular product with exceptionally high yield or tolerance to a harsh environment would also point in the direction of particular classes of genes and proteins. Intelligence or other reports may also include details on the purpose of release and/or development of a suspected GMO; - again pointing in the direction of particular classes of genes and proteins.

Detection of GMOs at knowledge levels 3 and 4 is unlikely to become routine in any GMO laboratory. Both the technology and workload required for sample analysis are limiting factors. Furthermore, taking samples for such analysis can require *ad hoc* sampling schemes, unlikely to be set up without *a priori* knowledge analysis (see also Ruttink *et al.* 2010b).

Detection of UGM at knowledge level 4 will probably be requested and/or required only in exceptional cases. It is foreseen that such work will be performed in selected laboratories and in coordination with e.g. the EU-RL GMFF and the competent authorities. Technological developments, availability of equipment, the nature of the sample, etc. will certainly affect the choice of analytical strategy in such a case. We therefore refrain from giving detailed guidance, and in stead refer to published literature (Nesvold *et al.*, 2005; Tengs *et al.*, 2007; 2009; 2010).

2.7 Control material for UGM

The construction of a GMO matrix is based on both theoretical and experimentally verified data on the presence or absence of genetic elements. Experimental verification means that control samples or certified reference materials have been used. The availability of control samples and certified reference materials is often limited for UGM, and especially for knowledge levels 3 and 4 the information provided in the GMO matrix is likely to be theoretical only. In order to increase reliability and harmonisation, it is desirable to establish a system for rapid and coordinated experimental verification of theoretical information in the (to be established) “GMO Reference Matrix” shared by the ENGL. See also annex 5.5.

3 Considerations for harmonized interpretation and reporting of UGM detection

3.1 General quality standards

A laboratory performing GMO analyses for enforcement purposes should be accredited according to ISO 17025 and follow agreed standards usually incorporated into accreditation schemes. Such quality standards imply that validated methods and certified reference materials are available. In the case of UGM, however, no specific detection method may be available or the validation of a particular method can be incomplete. Also, appropriate reference materials may not be available.

Note: The exceptions are – up to now - a few event and construct specific real-time PCR methods that can be used to demonstrate beyond reasonable doubt that UGM is present (e.g. methods for detection of Bt10 and E32 maize and LL601, Bt63, KMD1 and KeFeng6 rice (CRL-GMFF, 2006a; 2006b; 2008a; 2008b; Babekova *et al*. 2008; Reiting *et al*. 2010)), where corresponding reference material is available through the EU-RL GMFF.

Considering the absence of a solid reference framework, the interpretation and reporting of results on UGM presence should primarily focus on reliability. Convergence between datasets and ruling out or minimising the possible occurrence of false positives or negatives, are means to ensure reliability. For this, a brief general consideration on the latter aberrations is presented at first.

3.1.1 False positives (type I error)

The main criteria to be considered for a GMO detection method are its specificity (selectivity) and its sensitivity. A false positive occurs if the test result is positive (GM target or a specific GMO is detected) when the actual condition is negative (GM target or the specific GMO is absent). Laboratories under accreditation are obliged to take the necessary precautions to minimise the occurrence of false positives.

In the light of the matrix-approach, wherein the likely presence of a GMO is deduced from the combined presence of particular targets within the GMO, the evidence of linkage of the positive signals needs thus to be carefully weighted. Indeed, the presence of some quantity of GM material from an undeclared species is often a source of positive analytical

results that do not correspond to the results predicted from a declared list of ingredients. A typical example is a so-called “botanical impurity” in the form of GM soybean derived material in a “pure” maize product (Berben *et al.*, 2008). Therefore, it is of vital importance to determine from which species DNA present in the sample is derived.

Also, the DNA sequences of the promoters, terminators and coding sequences used as targets in GM detection methods are in most cases derived from and therefore present in an unmodified form in host organisms like viruses, bacteria, fungi, protozoans and invertebrates. Thus, in case the data analysis does not assign a set of positive signals to a GMO covered within the matrix, it is essential to rule out that these signals are caused by the presence of natural non-GM sources. Such “donor organism” specific controls should thus be part of the matrix approach when applying screening methods that target naturally occurring DNA sequences. Unfortunately, only few donor specific control methods are currently available (Cankar *et al.* 2005, Chaouachi *et al.* 2008, Weller *et al.* 2002, Wolf *et al.* 2000).

Preferably, any positive outcome for UGM presence should be supported by one or more confirmatory tests, e.g. DNA sequencing, to demonstrate that the source of the response is indeed a UGM. In this way, a sufficiently solid legal basis for a rapid alert action can be obtained. Positive results obtained with event specific or construct specific methods such as for the Bt10 and Event 32 maize or LL601, Bt 63, KMD1 and KeFeng6 rice (CRL-GMFF, 2006a; 2006b; 2008a; 2008b; Babekova *et al*. 2008; Reiting *et al*. 2010) would also provide appropriate evidence for the presence of UGM.

3.1.2 False negatives (type II error)

A false negative occurs if the test result is negative (GM target or a specific GMO is not detected) when the actual condition is positive (the GM target or the specific GMO is present). Evidently, the risk of false negatives is high for GMO at knowledge levels 3 and 4 because of the lack of appropriate information and/or suitable detection methods. However, this risk should be low for GMO at knowledge levels 1 and 2. Single nucleotide polymorphisms (substitutions) can negatively affect the performance of PCR detection methods (Ghedira *et al.* 2009) and have been reported for several GMOs (Bt176, MON 810, MON 863 and TC 1507 maize; Bertheau, 2005; Aguilera *et al.*, 2008; 2009; Holst-Jensen 2009b; Morisset *et al.* 2009) and reference genes (maize *adh1*, soybean *le1*; Broothaerts *et al.*, 2008; Holst-Jensen 2009b).

Application of screening methods in a matrix approach based UGM detection scheme introduces an additional challenge. Every screening method’s performance must be

determined for every GMO and potentially also against other realistic sources, independently of the theoretical presence or absence of the target. Otherwise, the analyst can draw the wrong conclusions when the theoretical distribution pattern is compared to the observed presence/absence pattern of target analytes. For example, both Bt176, MON 810 and Bt11 maize contain the *cry*IAb gene from *Bacillus thuringiensis* ssp. *kurstaki*, see e.g. CERA (2010), but the nucleotide sequences differ substantially between the three maize GMOs (modified codon usage, truncated and full length native gene, respectively). It is not unlikely that a screening method targeting the *cry*IAb gene will respond differently when applied to the three GMOs. This is exemplified with the inclusion of three *cry*IAb probes with different specificity in the GMO DualChip assay (Hamels *et al.*, 2009).

A second type-II error can be envisaged in the case the novel insert of a UGM consists of elements that are also present in the authorised GMOs in the sample.

3.2 Detectability

The ability to detect a target is influenced by sampling and the choice of analytical method(s). The practical LOD and LOQ (LOD_{pract}/LOQ_{pract}) is directly linked with the sampling and for analytical purposes it is defined by the quantity of analyte that is actually tested, e.g. the template DNA for PCR (Berdal and Holst-Jensen, 2001). The laboratory sample shall therefore be large enough to ensure that a satisfactory LOD_{pract}, respective LOQ_{pract} applies to the test. Zero tolerance for UGM will indirectly imply that the analytical method must be very sensitive, i.e. that the LOD/LOQ in UGM testing needs to be very low (see e.g. Regulation (EU) No 619/2011 (European Commission, 2011). If sampling or other factors do not offer the desired LOD/LOQ, this fact must be taken into consideration when the results are interpreted. Sub-sampling approaches (multiple parallel analyses) can be applied to improve the LOD/LOQ, but this can increase the workload significantly (Berdal *et al.*, 2008; Lee *et al.*, 2010).

3.3 Interpretation of results obtained with state-of-the-art technologies

In general, state-of-the-art technologies can only be applied to demonstrate the presence of UGM when dealing with GMO at knowledge levels 1 and 2, and exceptionally also at knowledge level 3. Event specific real-time PCR methods are the golden standard for unequivocal detection of any single-event GMO. For instance, the methods of analysis validated by the EU-RL in the context of the authorisation procedure and for the placing on the market, use and processing of existing products are event-specific quantitative methods. They are validated through a collaborative trial in accordance with the principles of ISO 5725

International standard and/or the International Union of Pure and Applied Chemistry (IUPAC) protocol. Construct specific methods, targeting fusion motifs that can not have a non-GM origin represent an alternative means of unequivocal detection of GMO, but can not be used to identify a particular GMO with the same certainty. A positive analytical result with an event specific method is reliable evidence of the presence of a particular unauthorised GMO, provided that the specificity of the method is confirmed through validation studies (collaborative or in-house) and that necessary controls (cf. International Organization for Standardization, 2006) give the expected results. If a construct specific method is used, and the construct is not found in any authorised event, a positive analytical result with such a method is also reliable evidence of the presence of unauthorised GMO. However, the identity is less certain. Again, sufficient specificity of the method must be demonstrated.

Quantitative real-time PCR methods allow the analyst to estimate the measurement uncertainty of the analytical result. This is particularly important at trace levels since analytical uncertainty increases with decreasing levels of GM material. Commission Regulation (EU) No 619/2011 (European Commission, 2011) sets as a Minimum Required Performance Limit (MRPL) the lowest level of GM material which is considered by the EU-RL for the validation of quantitative methods. This level corresponds to 0.1% related to mass fraction of GM material in feed and is the lowest level where results are satisfactorily reproducible between official laboratories when appropriate sampling protocols and methods of analysis for measuring feed samples are applied.For the interpretation of results obtained with a matrix approach and screening tests (Fig. 2), the following considerations should be taken into account before concluding on the possible presence of UGM. The absolute quantity of screening target detected (for positive tests) indicates whether false negative results for individual screening tests are likely. False negative test results can be a source of misinterpretation of results. The absolute quantity may be expressed as number of PCR forming units (*PFU*; Holst-Jensen & Berdal, 2004) observed in a test. The absolute limit of detection (LOD_{abs}) and quantification (LOQ_{abs}) are method specific performance characteristics established during method validation. Theoretically, the LOD_{abs} and LOQ_{abs} are 5-10 and 40-100 *PFU*, respectively, in a single PCR (Berdal & Holst-Jensen, 2001; Holst-Jensen *et al.*, 2003). As described in chapter 2.3.4 the degree of match between observed target patterns and patterns predicted for individual GMOs in the GMO matrix aids the analyst in determining which GMOs a sample is likely to contain. An observed pattern that can not be explained if only authorised GMOs are present indicate that UGM is present in the sample, but conclusive evidence will require additional confirmatory evidence. As a minimum, it must be demonstrated that the positive test result for target(s) responsible for the inexplicable pattern are not due to presence of a non-GM donor of the target (e.g. a

virus, bacterium or non-GM plant transfected by a natural virus or bacterium). If the tests lead to the conclusion that UGM is present, it is generally recommended to verify the test result with event or construct specific methods, or genome walking strategies such as anchor PCR (see annex 5.2.6) followed by DNA sequencing. In this way, the nature of the neighbouring sequences of the construct or of the event specific junction motif(s) could be identified and this can help in deciding on the origin of the signals. However, it can well be that no conclusive evidence can be obtained.

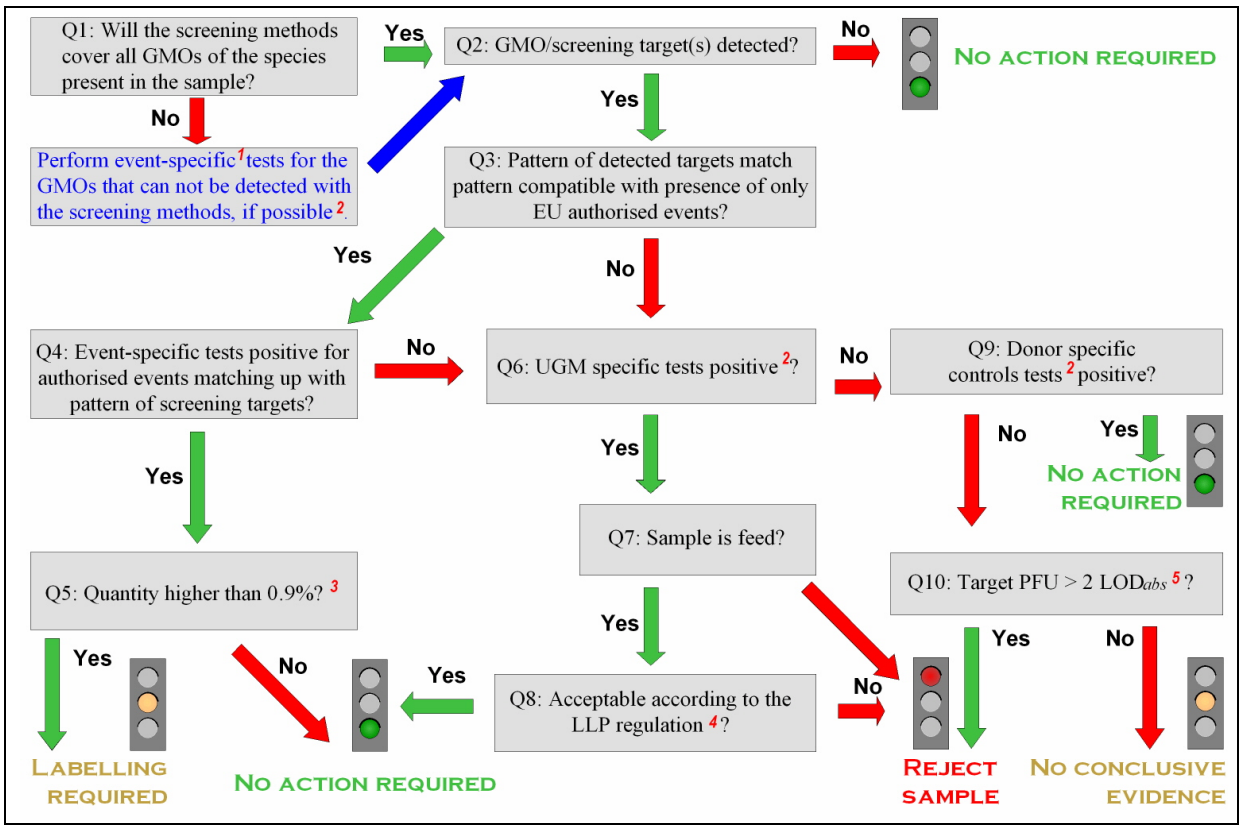

Figure 2. Decision tree for the application of the matrix approach to assess if UGM is present in a sample. *1*) In some cases where there is no event-specific method available, a construct specific method may be applied. *2*) It is recognised by the working group that sometimes no appropriate method is available. *3*) See specific details in Regulation (EC) No 1830/2003 on the labelling of products consisting of or produced from GMOs (European Commission, 2003b). Presence must also be adventitious and technically unavoidable. *4*) See Regulation (EU) No 619/2011 (European Commission, 2011) and the list of events for which the regulation applies (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). *5*) Although the observed number of target PFU is not formally quantifiable below the LOQ_{abs}, the working group is of the opinion that a signal equivalent to 2 x the LOD_{abs} (by extrapolation of the standard curve), is an acceptable signal threshold to avoid false negative test results (if the target is from the same GMO that produced the positive signal with one or more other tests). Options for additional analytical evidence of UGM presence (see section 2.6) can be applied if no conclusive evidence is obtained. However, the working group considers this to be for exceptional cases only, and to be outside the scope of the present document.

3.4 Reporting of results

Reporting of results related to UGM detection shall follow ISO 24276 and its associated standards ISO 21569, 21570 and 21572, EU regulations, recommendations and emergency measure requirements (European Commission, 2004; 2005; 2006b; 2006c; 2006d; 2008; 2011; International Organization for Standardization, 2004; 2005a; 2005b; 2005c; 2006) and other relevant *ad hoc* documents. A template for collection of sample and analysis related information in preparation of RASFF alerts has been prepared by the ENGL and is available to ENGL members. It is strongly recommended that the laboratory uses this template in its reporting to the competent authorities if the analytical results indicate presence of UGM in a sample.

4 Summary and conclusions

This overview provides in the view of the authors, the most complete reference on GMO analysis in the light of establishing a harmonised approach for GMO analyses, data interpretation and reporting. However, the field of GMO detection evolves rapidly and it is expected that considerable adjustments to the proposed strategies will be needed in the near future. This overview should thus be seen as a temporary state-of-the-art tool, requiring regular updating, preferably in collaboration with experts from outside the ENGL/EU. In line with this, the working group recommends that a new working group is established by the ENGL with a revised mandate that includes but is not limited to update the present overview. The working group feels that the present document is less of a guidance document than desired, and suggests that the guidance aspect is given particular attention by a new working group.

A first set of considerations have been deduced based on the enforcement experience with UGM presence when applying routine GMO testing. It has been a particular aim to avoid establishing a specific detection paradigm for UGM. No single assay has been identified as superior to alternative assays, despite a desire to achieve harmonisation among the ENGL members. However, the working group has agreed that initial screening applying the matrix approach, followed by *ad hoc* results verification using more specific PCR and/or DNA sequencing methods should be the common approach for routine GMO testing.

In the following, the working group wishes to highlight some remaining gaps and give some suggestions to facilitate the assessments and improve the reliability of conclusions on the absence or presence of UGM in food and feed products.

As a first consideration, it is apparent that the ability to conclude on the absence or presence of UGM is often hampered by a lack of information, of appropriate detection methods and of reference material. For this, it is considered essential that a GMO reference framework is established, e.g. through the availability of a “GMO Reference Matrix”.

Secondly, the availability of validated screening methods and appropriate reference materials is an absolute requirement to obtain data that are acceptable for enforcement actions.

As UGM are in most cases only present in low amounts in a product, interpretation tools will have to be made available that can deal with the uncertainty implied at these minute quantities of targets. However, it is also to be considered that without systematic efforts to trace UGM in products, it is likely that most UGM will not be detected at the threshold levels applied in routine analyses.

Finally, for broadening the transparency on decision heuristics, the development of decision support systems that are open to a large community e.g. through web applications is considered a step forward in such harmonisation. In this way, not only the decision criteria but also the analytical results and the data reporting could be harmonised in a very efficient way.

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5 Annexes

5.1 Available detection technologies and their applications

The following is an incomprehensive description of available detection technologies and their applications. Specific methods are not reviewed here, and the reader is in stead referred to methods databases such as the GMDD (Dong *et al.*, 2008) and published review papers (e.g. Holst-Jensen, 2009a; Stave, 2002, Van den Bulcke *et al*., 2007).

5.1.1 Protein based methods

Protein based techniques are also often referred to as immunological techniques because the detection is based on the immunological principle of conjugation between an antigen (the target) and an antibody (the probe specific to the antigen). Visualisation is usually done through a second antigen-antibody conjugate, in which a protein catalysing a colorimetric reaction is bound to the antigen-antibody conjugate of interest. Labelling can also be done using e.g. radioactive isotopes. Gel electrophoresis can be combined with staining without the use of immunological techniques. The specificity of antibodies vary. Monoclonal antibodies are identical, and hence highly specific to a particular epitope, whereas polyclonal antibodies are blends of antibodies specific to different epitopes on the target protein. If the UGM is “known”, then a monoclonal antibody can prove very useful, but otherwise polyclonal antibodies can have a higher potential for detection of the protein. The most commonly applied protein based methods are lateral flow strips (see 5.1.5), but other alternatives can be used for particular purposes.

5.1.2 2D-protein gel electrophoresis

Protein extracted from the sample can be separated in a two-dimensional pattern by iso-electric focusing and size based segregation, respectively. This distributes the proteins into a pattern where single proteins can be visualised and consequently detected. One of the advantages of this technique is that it can permit distinction between isoforms of the same protein, and combined with immunological staining, this can prove to be a useful tool for identification of UGM.

5.1.3 Western blots

Western blotting is based on one dimensional gel electrophoresis followed by blotting of the separated proteins to a membrane and identification of specific protein bands by probing

with specific labelled antibodies. The advantage of Western blotting is that the protein is identified by a combination of size and immunological conjugation.

5.1.4 ELISA

Enzyme linked immunosorbent assays (ELISA) are very popular and efficient tools for rapid detection of a particular protein. The antibody is immobilised on a solid support and the protein sample is added. If a protein with the antigen matching the antibody is present in the sample, then an antibody-antigen conjugate is formed. The reaction is then rinsed to wash off any unbound protein. A second antibody targeting a different part of the target protein is successively added, and if the protein is bound to the solid support via the first antigen-antibody conjugate, then the second antibody will bind. This second antibody is labelled, and the presence of the target protein can then be visualised.

5.1.5 Lateral flow strips

Lateral flow strips are related to ELISAs. The test solution is moving through a substance (matrix) driven by capillary forces, and the secondary antibody is mixed with the test solution immediately after the test solution has entered the filter. If the target protein is present, then the secondary antibody will bind to the target protein and move along with the protein and successively be captured by the primary antibody which is immobilised in a reading window. Lateral flow strips are widely used as screening devices for testing of large bulk samples of agricultural commodities for the presence of transgenic proteins. Unfortunately, these strips generally are not very sensitive, and their ability to discriminate between authorised events and UGM expressing the same trait is usually limited.

5.2 DNA based amplification methods

DNA can be amplified with several techniques. The most common technique is the polymerase chain reaction (PCR) technique, employing a thermostable DNA polymerase (see the following sections). Other techniques can be performed at ambient (room) temperature (isothermal; not further discussed here). All the amplification techniques involve denaturation of the double stranded nucleic acid followed by annealing of a short oligonucleotide (primer) and primer extension by a DNA polymerase. The nature of the primer, the number of primers involved, etc. varies from method to method. An mRNA (transcriptional product) can be used as a template after application of a reverse transcriptase and the synthesis of a copy DNA (cDNA). The cDNA is successively amplified like any DNA.

5.2.1 GMO element screening methods

GMO element screening methods are PCR based methods targeting genetic elements found in multiple GM events. The most commonly applied GMO screening methods are those that target the Cauliflower Mosaic Virus (CaMV) *35S* promoter (P-*35S*), the CaMV *35S* terminator (T-*35S*) and the *Agrobacterium tumefaciens* nopaline synthase (*nos*) terminator (T-*nos*). Other widely applied methods target marker genes from the cloning vectors (e.g. *nptII*, *bla*). The application of element screening methods as part of a matrix approach based analysis as described in chapter 2 is currently considered as the most cost-effective approach for UGM detection.

5.2.2 Gene (trait) specific methods

These are largely just an extension of the GMO element screening methods (see above) to cover targets that are less frequent in commercial events (e.g. genes encoding the most widely introduced traits: *cry*IAb*, cp4-epsps, pat*, etc.). However, methods targeting specific genes have also been developed and published in contexts outside GM detection. For example in the context of genetics, gene expression, phylogenetics and population studies. Many of these methods may not have been validated properly with respect to specificity, but they can prove useful in the context of UGM detection. Most of the genes introduced to GMOs were originally described from their original host species. In most cases, therefore, a detection method has been described prior to the transformation of the GMO. Challenges such as nucleotide substitutions, truncations and altered codon usage must be expected (e.g. for intellectual property right protection). The copy number of the target can vary both between and within events. The latter can result e.g. from segregation of multiple inserts in offspring.

5.2.3 Donor specific control methods

GMO element screening and gene (trait) specific methods both target DNA motifs derived from natural sources such as viruses, bacteria and plants. Positive signals observed with these methods therefore can stem from non-GM material. Donor specific control methods can be used to discriminate between samples positive for putative GM targets due to presence of non-GM donor derived material and samples positive due only to presence of GM material. Examples include methods to detect the CaMV, *Agrobacterium tumefaciens* and *Bacillus thuringiensis* (the donor of the *cry*-genes). Notably, the presence of material from the donor does not rule out the possibility that the presence of the target is also due to presence of GMO. The contrary, i.e. absence of donor, is usually a strong indication of GM

origin of the target (provided that false negatives can be ruled out, i.e. that the quantity of target observed is sufficiently high; see Fig. 2).

5.2.4 Construct specific methods

These are methods that target fusion motifs between the introduced elements, e.g. between a promoter and an enhancer element, a promoter and a gene, or a gene and a terminator. The constructs are often unique to a single event, but there are also several examples of the use of the same construct in the transformation of more than one GM event. This is particularly relevant in relation to UGM, as a single transformation experiment usually results in the generation of multiple transformants, each corresponding to a unique event. While further analysis, testing and breeding usually results in the marketing of not more than one of these events, several of the other co-transformants can persist as experimental events in the developing laboratory and its testing facilities. Escape or unintended release of such co-transformants is an important potential source of UGM. Construct specific methods can therefore be particularly useful if combined with other methods, e.g. an event specific PCR method (see below), possibly in combination with differential quantitative PCR (section 2.5.1.3). Construct specific methods target motifs that can be both single and multicopy in the haploid GM genome.

5.2.5 Event specific methods

These are methods that target motifs that are unique to a specific transformation event, and therefore exceptionally reliable for identification purposes. The target motif is usually but not always a fusion motif composed of a part of the recipient genome and a part of the inserted construct. In some cases, the target can be a rearranged motif, resulting from a unique rearrangement of the recipient genome or the inserted construct(s). The target is always single copy in the haploid GM genome, and therefore particularly fit for quantitation.

5.2.6 Fingerprinting/fragment profiling methods

These are methods that usually produce multiple fragments of various size, and the combined fragment pattern as observed by electrophoresis can be characteristic of (a) particular event(s). The profiles can be compiled in a database for later comparison with profiles obtained from test samples. Examples of profiling methods include the AFLP technique and its derivative anchor-PCR, as well as techniques in which short “random” primers are used (e.g. RAPDs, M13, microsatellites). Most of these techniques have found no application in GMO testing, because the number of different profiles obtained even for materials belonging to the same GM event is too many to be useful. However, anchor-PCR

yields few, relatively reproducible and unique fragments because fragments can only be amplified by the combined presence of the anchor-primer motif (a carefully selected sequence motif, usually presumed to be of GM-origin) and an adaptor-primer motif. A UGM is therefore likely to produce a unique anchor-PCR profile (Ruttink *et al.* 2010b).

5.2.7 Whole genome amplification

This is an amplification strategy that will amplify DNA in a non-targeted manner. The strategy is unfit for direct detection, but can produce large quantities of very pure DNA from minute samples. Studies indicate that the resulting DNA is fairly unbiased with respect to copy number ratios from template to final amplification product (Roth *et al.,* 2008). The DNA produced can successively be easier to study than genomic DNA, e.g. by targeted hybridisation techniques (Tengs *et al.*, 2007; 2010).

5.2.8 DNA sequencing

DNA sequencing is most commonly done on PCR amplified or cloned DNA fragments, but recent technological developments have made it possible to sequence complete genomes and transcriptomes (see e.g. Tengs *et al.*, 2009), although still at a high cost. The DNA sequence is the most complete information that can be obtained for a genetic modification. If the inferred sequence deviates from any authorised event and a non-GM origin can be excluded, then a DNA sequence is indisputable evidence of the presence of a UGM. Furthermore, the DNA sequence allows for assessment of risk by comparison against gene sequences with known biological functions. Publicly available DNA sequence databases include millions of gene sequences and can easily be exploited in similarity searches. These databases also include thousands of patented sequences, and are rapidly growing as new sequence information is made available by scientists all over the world.

5.3 DNA based hybridisation methods

The most commonly applied method for GMO detection is a combination of amplification and hybridisation, i.e. real-time PCR (see below) that also allows for quantitation of the target DNA.

5.3.1 Southern blots

Analysis of DNA fragments on a gel after electrophoresis, followed by blotting of the gel to a membrane and hybridisation of labelled probe to the blot has been applied for several decades. The specificity of the approach largely depends on the probe, and the technique

used to generate the DNA fragments (amplification technique or restriction endonuclease) can affect the resolution of the fragment profile. Varying the stringency conditions will affect the ability of the probe to hybridise, and low stringency can permit a probe to hybridise to fragments with DNA sequences that differ considerably from the probe sequence. This can facilitate the identification of UGM if a particular type of gene is suspected and e.g. PCR amplification fails to detect it due to minor substitutions in one or both primer sites. However, it can also result in false positives.

5.3.2 Probe based amplification methods including real-time PCR

This is essentially an extension of the amplification techniques described above, where a specific hybridisation probe is used to increase the specificity of the test. For quantitative PCR, real-time PCR is presently the by far most widely applied technique (but see also section 5.4). For confirmatory identification purposes, these techniques have a well documented record of reliable performance.

5.3.3 Low density targeted screening arrays

These are arrays of probes corresponding to specific amplification targets, as described above. The application consists of two steps, an initial amplification (usually by PCR) followed by hybridisation of the amplification products to the array and identification of probes to which product is bound, e.g. (Hamels *et al.*, 2009; Leimanis *et al.*, 2006; Morisset *et al.*, 2008; Xu *et al.*, 2006; 2007) The number of features (different probes and targets covered) on the array is typically < 100. At present the only multiplex assay that has been collaborative trial validated is the DualChip GMOchip v. 1.0 from EAT (Hamels *et al.*, 2009; Leimanis *et al.*, 2008). Notably, the present version commercially available, i.e. DualChip GMOchip v. 2.0, is an extended version and has been completely validated only internally by the company.

5.3.4 High density targeted screening arrays

These are arrays of probes corresponding to specific targets, where multiple probes represent each target. Gene arrays used to study gene expression is the most common and familiar type. For this purpose, the probes usually have perfect match with the gene in the target organism. However, for application in UGM detection, a novel approach was recently developed (Tengs *et al.*, 2007; 2010). Because the target in a UGM can contain several mismatches relative to the probes, the strategy applied to score positives is different from that applied in gene expression studies, and the targets are selected for the purpose of detecting introduced genetic elements rather than expressed mRNAs.

These arrays can only be applied to DNA with high relative concentration of the target element(s). This means that they can not be applied to samples containing mixed ingredients. Combined with whole genome amplication techniques, however, it is possible to apply these arrays to DNA from samples with low quantity of DNA (e.g. single grains).

5.3.5 High density profiling arrays

This is a theoretical concept first described by Nesvold *et al.* (2005). It is largely an extension of the previous concept. However, the probes are designed in an almost random manner, to increase the probability that any novel introduced sequence element shall yield positive hybridisation signal. The interpretation and further processing of the observed positive signals will require advanced use of bioinformatics, and successive characterisation e.g. based on anchor-PCR is necessary to verify that signals are not a result of natural intraspecific variation.

5.4 DNA quantification methods

Total mass of DNA can be quantified by measuring absorbance of UV-light at 260nm and the absorbance ratio at 260/280 nm to characterise the purity. Alternatives include the use of intercalating dyes such as ethidium bromide in combination with gel electrophoresis and SYBR Green or Pico Green in combination with a spectrophotometer. The latter approach is also sometimes used in real-time PCR as a substitute for a more specific hybridisation probe, thus allowing for measurement of the increased total mass of DNA in a PCR reaction, putatively resulting from amplification of a specific sequence motif.

5.5 Reference material availability

A wide range of reference materials are available, but their applicability is often limited by the availability of relevant information. Many of the reference materials based on plant materials are contaminated by traces of other GMOs (see certificates from IRMM and unpublished info from the EU-RL GMFF). Yet, the available reference materials can prove useful, e.g. to verify the performance of detection methods against specific GMOs (see also chapter 2 and the requirements for valid application of the matrix approach) or for establishing quantitative methods. Reference materials can also be used as a basis for sequence characterisation or verification of sequence information. Unfortunately, for many GMOs there is no reference material available.

An updated list of commercially available reference material is prepared every 6 months by the BVL in Germany. This list is available in .pdf format (see Fig. 3) from http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/nachweis_kontrollen/referenzmaterialien.pdf?__blob=publicationFile.

Referenzmaterialien für GVO Nachweis

Im folgenden sind die öffentlich verfügbaren Referenzmaterialien und deren Bezugsquellen aufgelistet aktuelle Materialien (farblich unterlegt) **Stand: 28.01.2009**

Nr.	Bezeichnung	Bestellcode	Hersteller/ Lieferant	Event	Unique Identifier	Organismus	Material	% GVO Gehalt	Menge je Einheit	Status des Referenzmaterials
1	Roundup Ready™ soybean	BF410a	IRMM / ERM	GTS-40-3-2	MON-04032-6	Soja	dried soya bean powder	<0,03	1 g	zertifiziertes Referenzmaterial
2	Roundup Ready™ soybean	BF410b	IRMM / ERM	GTS-40-3-2	MON-04032-6	Soja	dried soya bean powder	0,1	1 g	zertifiziertes Referenzmaterial
3	Roundup Ready™ soybean	BF410c	IRMM / ERM	GTS-40-3-2	MON-04032-6	Soja	dried soya bean powder	0,5	1 g	zertifiziertes Referenzmaterial
4	Roundup Ready™ soybean	BF410dk	IRMM / ERM	GTS-40-3-2	MON-04032-6	Soja	dried soya bean powder	1,0	1 g	zertifiziertes Referenzmaterial
5	Roundup Ready™ soybean	BF410e	IRMM / ERM	GTS-40-3-2	MON-04032-6	Soja	dried soya bean powder	2,0	1 g	zertifiziertes Referenzmaterial
6	Roundup Ready™ soybean	BF410gk	IRMM / ERM	GTS-40-3-2	MON-04032-6	Soja	dried soya bean powder	10,0	1 g	zertifiziertes Referenzmaterial
7	Bt-176 maize	BF411a	IRMM / ERM	E176 Maize	SYN-EV176-9	Mais	dried maize powder	<0,014	1 g	zertifiziertes Referenzmaterial
8	Bt-176 maize	BF411b	IRMM / ERM	E176 Maize	SYN-EV176-9	Mais	dried maize powder	0,1	1 g	zertifiziertes Referenzmaterial
9	Bt-176 maize	BF411c	IRMM / ERM	E176 Maize	SYN-EV176-9	Mais	dried maize powder	0,5	1 g	zertifiziertes Referenzmaterial
10	Bt-176 maize	BF411d	IRMM / ERM	E176 Maize	SYN-EV176-9	Mais	dried maize powder	1,0	1 g	zertifiziertes Referenzmaterial
11	Bt-176 maize	BF411e	IRMM / ERM	E176 Maize	SYN-EV176-9	Mais	dried maize powder	2,0	1 g	zertifiziertes Referenzmaterial
12	Bt-176 maize	BF411f	IRMM / ERM	E176 Maize	SYN-EV176-9	Mais	dried maize powder	5,0	1 g	zertifiziertes Referenzmaterial
13	Bt-11 maize	BF412a	IRMM / ERM	Bt11	SYN-BT011-1	Mais	dried maize powder	<0,012	1 g	zertifiziertes Referenzmaterial

Figure 3. View of part of list of commercially available reference materials for GMO detection. The list is prepared by the BVL in Germany. Materials added to the list since the previous version are highlighted in green. The date of preparation of the published version is indicated in the upper right corner.

European Commission

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Abstract
Unauthorised GMOs (UGM) and derived materials are not uncommon in products found on the European market. At present there is zero tolerance for UGM in the EU. In most documented cases, the UGM concentration relative to the product in which the UGM material is found, was low. Low level presence will always represent a challenge to analytically based detection, in particular if the UGM is obscured by other GM material. Analytical ad hoc implementation of the zero tolerance for particular UGMs in feed is described in Regulation (EU) No 619/2011. In Europe, GM detection is predominantly achieved with polymerase chain reaction (PCR) derived methods targeting the transgenic construct and insertion site DNA sequences. The increase in number and divergence of GMOs developed and commercialised has gradually forced the GM detection laboratories to rationalise their analytical work, and most laboratories now apply initial PCR based screenings followed by (when appropriate) more specific PCR based identification and quantification.
The detection of any GM is dependent on availability of suitable detection method(s) and control materials to verify the performance of the method(s). Other information, e.g. describing the novel trait, introduced genetic elements, etc. may also facilitate detection, verification and identification of the GM. For UGM, this is a major challenge, and the GMOs are therefore classified into four knowledge groups in the present document. This classification may facilitate stakeholder communication and decision making in analytical laboratories.
A decision tree is presented, summarising the recommended principles of GM and UGM detection. Notably, the state-of-the-art of GMO analysis is not static, and it is expected that the guidelines and recommendations presented in this document will have to be modified on a regular basis. Finally, the document highlights a number of R&D priorities and points out the need for reinforced information sharing at the global level.

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