Detection of genetically modified plants

– methods to sample and analyse GMO content in plants and plant products.

By Mette Lübeck

Preface.

The cultivation of genetically modified (GMO) plants is becoming increasingly important worldwide and transgenic crops are utilised on a large scale in North America and several other areas. The national governmental bodies and the general public concern for the use of GM plants have acted differently in Europe than in North America. This has led to differences in approvals of GM plants and only few of the genetically modified plant cultivars approved elsewhere are approved in the European Union.

The GMO-area is totally harmonised in the EU by directive 90/220/EC on the deliberate release into the environment of genetically modified organisms. The directive is implemented into Danish legislation by the "Act on Environment and Genetic Engineering" no 356 from June 6th, 1991. Adopted in accordance with the Danish Act are a number of statutory orders *e.g.* "Statutory Order on Transport and Import of Genetically Modified Organisms (GMOs)". A new directive, 2001/18/EC on the deliberate release into the environment of genetically modified organisms, repealing directive 90/220/EC, has been adopted and will be implemented in Danish legislation by October 2002. In addition, Denmark is committed by an EU Action Plan on GMOs, that Denmark endorsed in September 2000 in EU's Standing Committee on Seed and Propagating Material for Agriculture, Horticulture and Forestry. The main objective of the Action Plan is to meet growing conditions and other requirements for purity concerning the adventitious presence of GMOs in conventional seeds. Thus the obligation for the Danish inspection authorities is to make sure that non-EU-approved GMOs are not imported from third countries or enters the EU as impurities in conventionally grown crops.

The inspection of GMO transport is achieved at present by documentary inspection alone. Inspection of imported seed is done by controlling lists of import from the central customs and tax administration. If however, import of tomatoes, cotton, soybeans, maize, beets or oilseed rape occurs, it is determined on a case-by-case basis whether the imported batch will be analysed for the presence of GMOs.

The aim of this report is to uncover and overview existing methods for sampling and analysing GMOs. The report focuses on identification of the current problems in the inspection area, *e.g*. how to make the distinction between non-approved and approved GMOs, and links these to the accessible methods. Since the inspection obligations in practise will mainly involve imported seeds (primarily from non-EU-countries), most attention has been devoted to this area.

The preparation of the present report has been overseen by a steering committee, which consisted of Helle Haugaard, the Danish Forest and Nature Agency, Svend Pedersen and Michael Krause, The Danish Plant Directorate, and Folmer Eriksen and Jan Petersen, The Danish Veterinary and Food Administration.
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1. Introduction.

A range of transgenic plants are already approved or under approval internationally. Only few of these genetically modified (GMO) plants have been approved in the European Union. This poses a problem for inspection authorities to ensure that the non-approved GMOs (genetically modified organisms) are not imported into the EU either as constituents of feed and food products or as "GMO-impurities" in products of the food chain. It is thus necessary to be able to distinguish GMOs from non-GMOs and to determine whether a specific GMO is EU-approved.

The aim of the present project is to uncover and overview existing methods for sampling and analyses of genetically modified plants and to uncover to what extent data is accessible for an unambiguous identification and quantification of GMOs.
A GMO is defined in Council Directive 2001/18/EEC as an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. The genetic modification occurs at least through the use of the following three techniques:
1) Recombinant DNA techniques using vector systems;
2) Techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;
3) Cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

Genetic modifications are carried out by the insertion of several smaller pieces of DNA from various sources, into the genome of the plant to be modified. A gene construct consists typically of three elements: 1) The **promoter** functions as an on/off switch for when and where the inserted/modified gene is active in the recipient plant. 2) The inserted/modified **gene** (structural gene) encodes a specifically selected trait. 3) The **terminator** functions as a stop signal for transcribing the inserted/altered gene. In addition marker genes for distinguishing GMOs from non-GMO during crop development may be present as well as residual DNA material from insertion plasmids.

At present, the vast majority of the existing GM plants have been developed in order to tolerate herbicides or possess resistance towards insects and viruses. Other genes inserted in GMOs, encode different agronomic traits such as delayed ripening and delayed softening of *e.g*. tomatoes and change in oil composition in *e.g*. soybean. Many of the genes inserted in various crops are derived from the same source. For example, a gene encoding the 5-enolypyruvylshikimate-3-phosphate synthase (EPSPS), resulting in tolerance to glyphosat-herbicides, from the CP4 strain of the bacteria *Agrobacterium tumefaciens* has been inserted in oilseed rape, soybean, maize, cotton, potato and sugar beet. The first transgenic plants on the market had single traits inserted, but there is a tendency that more transgenic varieties have several traits incorporated.

In plants, particular genes have been identified, cloned and transferred into particular parental lines, which have then been used as gene donors or carriers in backcross breeding programs. New inbred lines or parental lines often referred to as

genetically modified lines are then used in the development and production of genetically enhanced hybrids. A list of genes used for the currently internationally registered GM plants are given in Appendix 1.

The countries with largest areas of GM crops are USA, Argentina, Canada, China, and Australia in that order. These countries alone accounted for 99 % of the total GMO-growing area in 2000. The transgenic crops, which are cultivated over the largest area, are soybean (2000: 25.8 mill. ha.), maize (2000: 10.3 mill. ha.), cotton (5.3 mill. ha.) and oilseed rape (2.8 mill. ha.) (James, 2000).

For inspection of GMOs, the following different cases are considered:
1) EU-approved GMOs, 2) conventional and 3) organic grown seed, feed and food products for which different aspects in relation to GMOs are considered:

1) For **EU-approved GMOs** (Appendix 2), it is important to confirm the correct trait as specified on the label or in associated documents and to ensure that the product is not contaminated with non EU-approved GMOs. At present only few EU-countries grow these crops.

2) For **conventional products** it is permissible to have a GMO level of up to 1% of the EU-approved GMOs, while no non-EU-approved GMOs (Appendix 3) may be present (*i.e*. zero level) according to the Commission Regulation (EC) No 49/2000.

3) For the **organic products**, GMO present are not permitted at all; thus a zero level is required.

The Standing Committee on Seed and Propagating Material for Agriculture, Horticulture and Forestry has adopted an Action Plan for adventitious presence of GM seeds in seed lots of conventional plant varieties. A working document has been prepared as a proposal for changes of the EU seed marketing directives (Council Directive 66/400/EEC and amendments included in 98/95/EEC). In this document, it is stated that it has to be distinguished between self-pollinating and vegetatively propagated crops and cross-pollinating crops. In both cases, initial impurities of GM seeds may lead to significantly higher levels in the harvested product, thus exceeding the established 1 % threshold level. Therefore, the suggestion is to have a seed tolerance threshold of 0.3% in the case of cross-pollination crops, and of 0.5 % in the case of self-pollinating and vegetatively propagated crops. For soybeans and field pea, for which the probability of dormant seeds, which germinate after some years, is very low, a seed tolerance threshold is suggested set at 0.7 %.

These levels of acceptable GMO-content account for EU-approved GMOs. When it comes to non EU-approved GMOs, these should not be present in a seed lot. However, it is the intention to amend Directive 2001/18/EEC to provide for the possibility of establishing thresholds for technically unavoidable or adventitious presence of minute amounts of GMOs. Thus, the zero-level – which in practice is impossible – is by the Scientific Committee on Plants suggested set at a level of 0.1 %, in accordance with the lowest scientifically defensible threshold for quantification of GMOs using PCR based methods (see Section 3.2.1.2).

Therefore, it is necessary to be able to determine whether a specific GMO is EU-approved and to be able to quantify the amount of (EU-approved) GMOs present. Detection and identification of GMOs requires that specific information of the inserted DNA is available. For the EU-approved GMOs this information is available and methods for detection are being elaborated. For many of the non-EU-approved GMOs, the exact information is difficult to find.

1.1 EU-approved GMO

It is stated in Annex 3 of the revised Council Directive 2001/18/EEC on the deliberate release into the environment of GMOs, that the notifier shall provide the necessary data and techniques for detection and identification of the GM plant. In addition, Annex 4 of the Directive describes the additional information to be provided on the genetic modification for the purpose of developing one or several registers, which can be used for the detection and identification of particular GMO products to facilitate control and inspection. A list of EU-approved GMOs is given in Appendix 2.

Data for EU-approved GMOs will thus be accessible from the notifier's application and included in a database containing information about the relevant sequences for the EU-approved GMOs. The European Commission Joint Research Centre and the German Federal Institute for Health and Consumers Protection (BGVV) are about to complete databases, containing information about the relevant sequences for the EU-approved GMOs.

1.2 Non-EU-approved GMO

Data for the non-EU-approved GMOs can be found through different sources. Of the GM-crops grown worldwide, most have been developed and approved in USA (APHIS-granted). GM-crops grown in many other countries (*e.g*. Canada, Japan, Mexico, Argentina, Australia and China) are mostly based on US patents and technologies, though some of them have been further developed in order to adapt to local conditions. Through the home page of USDA/APHIS it is possible to obtain rather detailed information about specific inserted genetic material.

Overview of information about specific GMOs and their approval status can be found through the OECD Biotechnology Product Database and the Canadian Agbios database: http://www.olis.oecd.org/bioprod.nsf and http://www.agbios.com/dbsearch.asp. However, this information does not specify the sequences of the inserts and this information is difficult or impossible to find. One of the reasons is that the sequences can be proprietary of the company who developed the plant variety and thus not commonly available. Internationally approved GMOs including the EU-approved GMOs are listed in Appendix 3.

1.3 GMO Detection

GMOs can be identified by detecting either the inserted genetic material at DNA level, the mRNA transcribed from the newly introduced gene, the resulting protein,

metabolite or phenotype respectively. The analytical tests on raw materials, as *e.g.* seeds, are generally carried out with the polymerase chain reaction (PCR method) detecting the inserted DNA, immunological assays detecting the resulting protein (*e.g.* the enzyme-linked immunoassay (ELISA) and lateral flow sticks), or using bioassays to detect the resultant phenotype (*e.g.* herbicide bioassays). Although much progress has been achieved in the development of genetic analysis methods, such as those based on the use of PCR, several other analytical technologies that can provide solutions to current technical issues in GMO analysis are emerging. These methods include mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices and, in particular, DNA chip technology (microarrays).

So far only PCR has found broad application in GMO detection as a generally accepted method for regulatory purposes.

1.3.1 Criteria for analytical methods.

The essential characteristics of a strong GMO analytical method are the following:

- It must detect all GMOs.
- It must provide quantitative information on how much GMO content is present.
- It must deliver informative results with a wide range of foods and agricultural products.
- It must deliver maximal reliability and reproducibility, and must avoid false positives and false negatives.
- It must be sensitive and reliable enough to obtain exact results in all control laboratories.

This makes a demand for validation of reference methods for suitability and certainty as discussed in see section 4.

It is important to differentiate that assessment of GMO content in samples can be divided in three different levels:

1) **Detection.** The purpose of detection is to determine whether a sample contains GMOs. For this objective, a screening method can be used resulting in a positive/negative statement. The screening methods are usually based on the polymerase chain reaction (PCR).

2) **Identification.** If there is a positive detection of GMOs, further analysis is required to discover which GMO it is and thus whether the GMO is approved within the EU. The only analytical methods, which unequivocally may enable identification of each GMO variety are methods based on PCR.

3) **Quantification.** If a product has been shown to contain GMO(s), the next step is to assess compliance with the 1% threshold level (or the 0.3 or 0.5% level, respectively for seeds) by the determination of the exact amount of each of the GMOs present in the sample. Typically quantification is performed using semi-quantitative PCR or Real-time PCR.

These levels can be carried out in a step-wise approach. However, different companies and laboratories may have different strategies for carrying out the three different levels.

2. Sampling

2.1 Sampling plan

It is important to note that no matter which analytical method is used, application of the correct sampling method is crucial for the result. In general, for quantification of a GMO contamination there is three main sources of error: 1) the sampling scheme, 2) the testing plan (how to handle the submitted sample) and 3) the accuracy and precision of the analytical method. GMO contamination may not be evenly distributed through a seed lot or a field. Each seed or plant either is or is not genetically modified and too small a sample will therefore not be statistically representative. When sampling in a seed lot or a field, it is sampling of discrete populations, *e.g*. one seed in 1.000 is 0.1% of the total. Some recommend seed samples of approximately 1.500 to 3.000 grams to detect tiny contamination levels, while other recommend a certain number of seeds based on the mass equivalent (*e.g.* 10.000 seeds, see below). The sample size will depend upon what detection level is important and what one is looking for.

Assurance that the sample is representative of the larger lot of material, from which it is taken, depends on two factors:
1) The sampling plan: the sample, termed "**field sample**", must be taken in a manner that insures that it is statistically representative of the larger lot of material, and the field sample must be of sufficient size to allow analysis to the desired sensitivity.
2) The sample that is analysed in the laboratory termed "**analytical sample**" must be representative of the field sample submitted for the analysis.

In order to use standardised methods for sampling of seed, the international seed testing agency (ISTA) has developed a sampling guide "ISTA Handbook on Seed Sampling (1986)" (information on this book, see http://www.seedtest.org/Cartease/item-detail.cfm?ID=0.10&storeid=1). The EU-network of inspectors: European Joint Enforcement Group on Deliberate Release of GMOs (EEP-DR) has also discussed this matter and has developed Standard Operating Procedures (SOPs) for both sampling in fields and sampling of seeds. Principles from these SOPs are summarised below.

2.1.1 Principles for sampling for GM inspections of fields

Sampling should be done from plants randomly distributed throughout the crop and not biased towards one part of the field or area. A 'W' walk through the crop would provide a suitable spread of sampling throughout the crop. Furthermore the location of the plants to be sampled should be marked on the trial plan and the plants marked or recorded in some way so their location can be found later if necessary. For actively growing plants one leaf should be removed from near the top of the plant and placed in a clean, new plastic bag, using disposable gloves – it is, however, not necessary to use different bags for different leaf samples.

The number of samples taken to check contamination will depend on the level of contamination suspected or level of statistical assurance that is required. Some examples of sizes of samples required are:
100 plants give a 95% confidence limit of detecting a 3% contamination level
200 plants give a 95% confidence limit of detecting a 1.5-% contamination level
300 plants give a 95% confidence limit of detecting a 1-% contamination level
3000 plants give a 95% confidence limit of detecting a 0.1% contamination level

2.1.2 Principles for sampling for GM seed in seed lots

Seed should be sampled according to Article 7 of Council Directive 66/400/EEC on the marketing of beet seed and from seed lot sizes as defined in Annex 2 of the same Directive. The general principles and methods should be in accordance with the ISTA rules (1999) and the associated ISTA Handbook of Seed Sampling mentioned above. The size of the submitted sample shall be determined according to the requirements for the testing methods used for GMO detection in seed certification. The working sample is a sub-sample of the submitted sample prepared in the laboratory according to ISTA methods. It shall contain a minimum of 3000 seeds.

Working groups under the Standing Committee on Seed has worked on a harmonisation of guidelines on sampling procedures of seed lots for production of representative samples for GMO-analysis and identification of suitable analysis methods. They have elaborated different documents concerning testing plans, sequential tests and statistical aspects of GMO detection in conventional seed lots. Sequential tests, *i.e.* to perform the planned tests in steps, are suggested as some of the efforts needed in order to save work and minimise the cost of tests. At each step a decision is taken whether to reject the seed lot, to accept the lot, or to conclude that more tests has to be performed.

One of the main concerns of the Standing Committee on Seed and Propagating Material for Agriculture, Horticulture and Forestry based on different opinions from experts from the different countries was whether the GMO-content should be calculated as a DNA-% or as single seed-%. Three different suggestions to sampling procedures and analyses were as follows:
- Sequential analysis of 3.000 seed divided into subsamples using qualitative PCR
- DNA-% analysis of 3.000 seed using quantitative PCR
- Single seed analysis of 3.000 seed following germination using qualitative PCR.

The opinion of the size of seed in samples also differs. The French National Validation Organisation (AFNOR) has in the document XP V 03-020-1 stated that the size of laboratory samples depends on the type of product and in particular on the size of particles and on their homogeneity. For *e.g.* seeds the minimum laboratory sample size is given by AFNOR as 10.000 grains or their mass equivalent.

2.2 Critical parameters in connection with sampling and sample handling

Sampling procedures and the procedures used to prepare the analytical sample from the field sample thus are keys to reliable and informative analysis of foods and

agricultural products for GMO content. This aspect of analysis is often forgotten when laboratories develop GMO analytical services.

If samples are to be analysed by a commercial company, it is very important to ask very specifically how the laboratory handles samples. Many labs will analyse only a small portion (not statistically representative) of the sample submitted for analysis, regardless of the size of the original sample that they received. Small sample sizes increase the possibility for false negatives (*i.e*. finding a sample as GMO-free even it actually contained such material). Thus a sample obtained via a valid sampling plan may be improperly handled by the laboratory, resulting in an analytical result that does not accurately reflect the GMO content of the sample submitted for analysis or the GMO content of the larger lot of material from which the sample was taken.

In addition it is important to realise that repeated analytical samples drawn from a homogenised field sample may not have identical proportions of GMO versus non-GMO copies especially when tiny GMO levels is present. The statistics for this problem is described in Kay & Van den Eede (2001).

It is also important to consider aspects of cross-contamination when preparing samples. For example when grinding large samples, the sample preparation area requires environmental control. Several of the analytical methods are very sensitive and dust drift can cause cross-contamination (false positive). Also the sampling device (grinding and mixing equipment) must be analytically clean and free of material before collecting the next sample. Just a few GMOs left in the device will cause a positive result in the next sample.

3. GMO testing methods.

It is important to realise that the analytical methods differ in many levels. Only PCR offers at present a way for performing a general screening for GMOs and detection of particular “events”. Phenotypic characterisation and immunoassays detect particular traits that may be present in several GMO types (*e.g*. the Cry1a protein and genes, conferring herbicide tolerance, are present in a range of different GMOs).

In the following, the three commonly used methods, herbicide bioassays, immunoassays and PCR and the most promising of future methods, microarrays are described and compared. Herbicide bioassays and immunoassays can be regarded as “low-technology methods” because they can be set up in most laboratories while PCR and microarrays are regarded as “high-technology methods” requiring more equipment and trained specialists. A list of companies that either offer to analyse samples for GMO-content or sell GMO testing kits can be found in Appendix 4.

3.1 Low-technological methods.

3.1.1 Phenotypic characterisation (herbicide bioassays).

Phenotypic characterisation allows detection of the presence or absence of a specific trait. So far only tests for traits as resistance or tolerance to herbicides are available. Such tests can be used to test for presence or absence of herbicide resistant GMO varieties and is termed herbicide bioassays. They consist of conducting germination tests on solid germination media in the presence of a specific herbicide, where non-GMO and GMO seeds show distinct characteristics. The detection level is dependent on germination of the seed and the germination methods should ensure that all viable seeds of the tested sample germinate. Seeds tested positive should be exposed to subsequent tests for confirmation.

The herbicide bioassay tests are claimed to be accurate, inexpensive, and useful as a preventative test primarily for seed companies. Companies are using the herbicide bioassays to check individual shipments as a quality assurance program. Negative trait and positive trait seeds should be included as controls with every sample testing.

At the moment herbicide bioassays are available for Roundup Ready soybean, maize, cotton and oilseed rape, and Liberty Link maize (see Appendix 4). In the future bioassays for insect-resistant or other GMO varieties may be developed.

Typically a test of 400 seeds is carried out. Depending on the size of the seed lot or if the germination level is low, this amount can be increased to 1.000 to 2.000 seeds/seed lot. Detection level and quantification depend on how many seeds that are tested. Some commercial companies offering seed test analysis based on herbicide bioassays are listed in Appendix 4.

3.1.2 Protein methods.

Immunoassay is the current method for detection and quantification of new (foreign) proteins introduced through genetic modification of plants. The crucial component of an immunoassay is an antibody with high specificity for the target molecule (antigen). Immunoassays can be highly specific and samples often need only a simple preparation before being analysed. Moreover, immunoassays can be used qualitatively or quantitatively over a wide range of concentrations. Similar to herbicide bioassays, immunoassays require separate tests for each trait in question.

Making a valid identification of the foreign protein in GMOs using immunoassays depends on the availability of the particular proteins for development of the antibodies, which is the essence of the assay. The proteins can be proprietary of the company who developed the plant variety and thus not commonly available. Furthermore, the likelihood of development of a successful immunoassay depends on certain characteristics of the antigen used for development of the antibody, *i.e.* size, hydrophobicity and the tertiary structure of the antigen.

The antibodies can be polyclonal, raised in animals, or monoclonal, produced by cell cultures. Commercially available polyclonal antiserum is often produced in rabbits, goats or sheep. Monoclonal antibodies offer some advantages over polyclonal antibodies because they express uniform affinity and specificity against a single epitope or antigenic determinant and can be produced in vast quantities. Both polyclonal and monoclonal antibodies may require further purification steps to enhance the sensitivity and reduce backgrounds in assays. The specificity of the antibodies must be checked carefully to elucidate any cross-reactivity with similar substances, which might cause false positive results.

Immunoassays are utilising the specific binding of the antibody to the antigen. Thus, the availability of antibodies with the desired affinity and specificity is the most important factor for setting up test systems. The reaction between the antigen and antibody is detected through a second antibody preferably reacting with another epitope on the antigen. The second antibody carries a label that can be detected or can generate a detectable signal.

Different immunoassays are available suited for field use as well as for well-equipped laboratories.

3.1.2.1 ELISA (enzyme linked immunosorbent assay).

In ELISA the antigen-antibody reaction takes place on a solid phase (microtiter plates). Antigen and antibody react and produce a stable complex, which can be visualised by addition of a second antibody linked to an enzyme. Addition of a substrate for that enzyme results in a colour formation, which can be measured photometrically or recognised by eye (Figure 1).

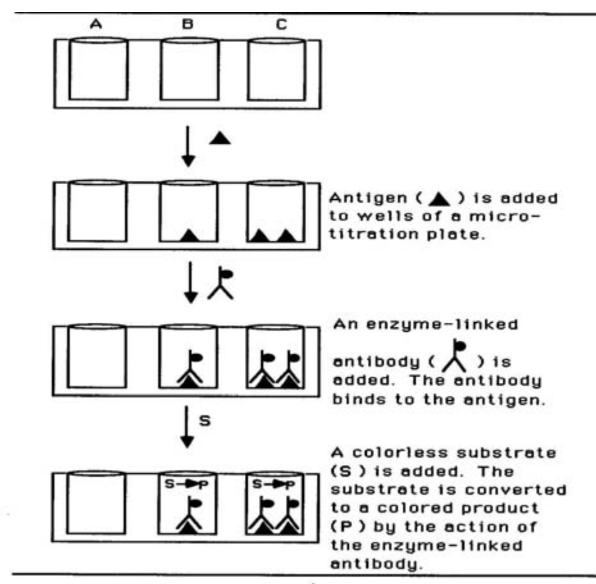

Figure 1. Schematic presentation of the ELISA principle.

Some ELISA plate kits are supplied with calibrators (known concentrations of the target analyte in solution) and a negative control (known to be free of the target analyte) for both visual and instrumental interpretation of the test results. These standards (calibrators and control) exhibit distinctly different shades of blue colour at the different concentrations provided (for example: zero, 0.5 ppb (parts per billion), 1 ppb, 5 ppb, 10 ppb). By comparing the colour of the sample against the standards, it is possible to visually determine the concentration range of the sample, for example, "between 1 and 5 ppb". This interpretation is semi-quantitative. Alternatively, quantitative interpretation can be performed by inserting the microwells in a "microplate reader", which precisely measures the optical density of all samples and

all standards at the same time. Using software provided with the reader, the user then calculates the sample concentration from the standard-curve.

ELISA test kits provide the quantitative results in hours with detection limits less than 0.1%. However, some companies operate with slightly higher quantification levels as *e.g.* 0.3%.

3.1.2.2. Lateral Flow Sticks.

Dipstick formats (lateral flow sticks) can be used to detect genetically modified organisms (GMOs) in leaves, seeds and grains. Paper strips or plastic paddles are used as support for the capture antibody and this is then the reaction site. The strip/paddle is dipped in vials containing the different solutions. Each dip is followed by a rinsing step. The final reaction includes a colour change in the vial, where the strip/paddle is placed. Recent development of dipstick format has led to lateral flow techniques where reactants are transported through the channels of a membrane by capillary forces. One single step is enough for performing the assay, and controls for reagent performance are included. Antibodies specific to the foreign protein are coupled to a colour reagent and incorporated into the lateral flow strip. When the lateral flow strip is placed in a small amount of an extract from plant tissue that contains foreign protein, binding occurs between the coupled antibody and the protein. A sandwich is formed with some, but not all the antibody that is coupled to the colour reagent. The membrane contains two capture zones, one captures the bound foreign protein and the other captures colour reagent. These capture zones display a reddish colour when the sandwich and/or non-reacted coloured reagents are captured in the specific zones on the membrane. The presence of a single line (control line) on the membrane indicates a negative sample and the presence of two lines indicates a positive sample (Figure 2).

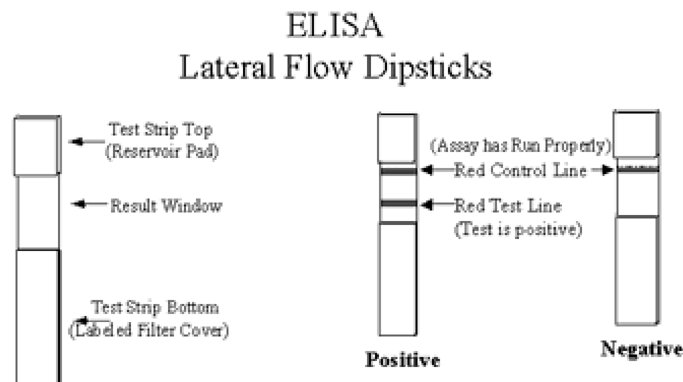

Figure 2. Schematic drawing of lateral flow sticks

Lateral flow techniques are qualitative or semi-quantitative. By following appropriate sampling procedures, it is possible to obtain a 99% confidence level of less than 0.15% GMO for a given lot.

There are several commercial sources of test kits, both ELISA and dipstick formats. Examples of providers are given in Appendix 4.

3.2 High technological methods.

3.2.1. PCR

PCR (polymerase chain reaction) is the most widespread method for identification of GMOs. PCR consists of extraction and purification of DNA, amplification of the inserted DNA by PCR (Figure 3) and confirmation of the amplified PCR product. In principle, PCR can detect a single target molecule in a complex DNA mixture. However, it is important to recognise the difference between the theoretical sensitivity of the PCR method and actual sensitivity of PCR-based assays for detecting GMOs. Detection limits of GMO detection assays are treated in section 3.2.1.3. PCR-based assays require other steps that profoundly influence the reliability and sensitivity of the method as a whole. Among these are sample and sample preparation, which are discussed in section 2.

Each step of the PCR influences both the reliability and sensitivity of the assay and should be optimised in order to develop a high-quality GMO analytical method. The significant challenges in carrying out PCR analysis are to successfully extract DNA from the sample with high yield (optimal is 100%), to avoid DNA degradation, and to remove chemical contaminants that might inhibit PCR amplification. Furthermore, PCR must include proper controls and standards that facilitate verification for each analysis to ensure that the method is operating optimally, thereby verifying the reliability of results obtained. This includes access to relevant reference material (see section 4) and protocols for the testing material.

Most PCR based GMO analysis includes a positive control primer set, which is specific for a gene that is present naturally in all varieties of the applicable crop. For instance, when analysing *e.g*. a soybean sample, the positive control is specific for a gene present in all soy varieties, *e.g*. the lectin gene, whether conventional or transgenic. This primer set is specific for a “species-specific reference gene” and is used in the analysis of all samples. If a strong signal with the positive control primer set is not observed in an analysis, the integrity of the DNA is called into question, or alternatively the presence of a substance in the DNA preparation that is inhibitory to PCR is likely.

PCR tests can be designed to detect any of the inserted genetic material: promoter, structural gene, stop signal or marker gene. The exact design of any particular test depends on the requirements. PCR can be used for a **general screening of GMOs** using primers that recognise common DNA, which most GMOs harbour, for example the commonly used Cauliflower Mosaic Virus (CaMV) 35S promoter or *Agrobacterium tumefasciens nos* promoter, or the *nos* terminator.

PCR can also be used to detect and identify specific GMOs more precisely. However, making a valid and unique identification by using PCR requires information about the inserted sequences. For a positive unique identification it should be related to the specific transformation event of the GMO. The only unequivocal strategy is to use plant-construct junction sequences as primer targets. This gives indirect information of the whole inserted sequence including the used promoter, active gene and enhancer/ terminator. The methods are described in more detail below. There

are several commercial sources of PCR test kits as well as companies that offer GMO testing of samples (Appendix 4).

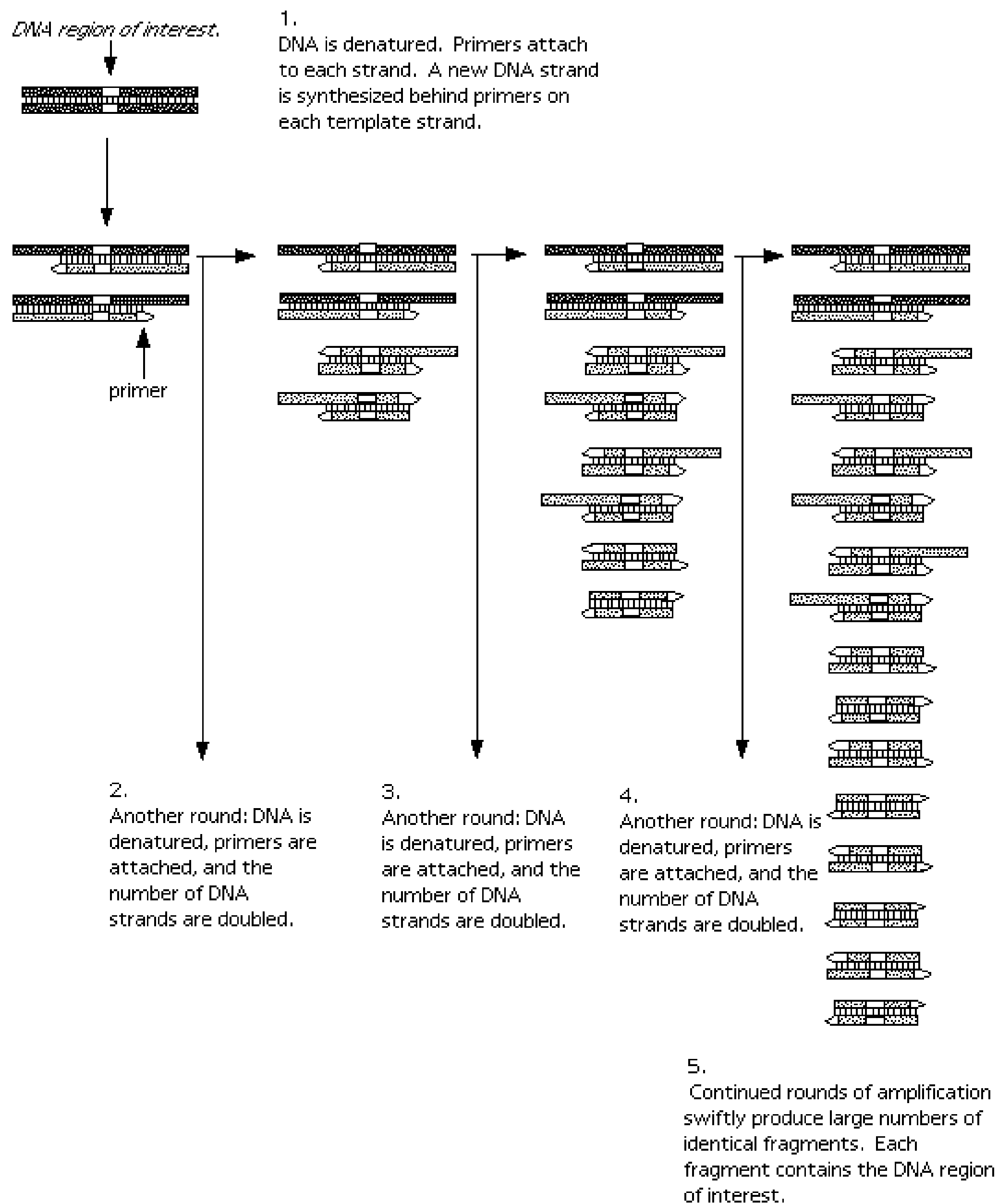

Figure 3. Schematic presentation of the principle of the polymerase chain reaction.

3.2.1.1 Qualitative PCR analysis

As described above, the essential for GMO detection by PCR is the choice of target for the primers. In principle, there are three different strategies for choosing an appropriate target:

1) <u>The use of genetic elements</u> commonly used in GMOs (the 35S or *nos* promoter, the *nos* terminator, the kanamycin resistance gene *npt*II). This is called a **general**

screening of GMOs and allows the suspicion of the presence of GMOs in case of a positive response. For example the 35S promoter of CaMV is *e.g*. currently found in 32 GMOs (Appendix 3).

2) To detect a particular gene construct, *i.e*. the junction sequences between two adjoining DNA segments can be the target for a specific detection of the genetic construct. However, these two joint elements can be introduced into other organisms resulting in different GMOs containing the same genetic construct. Consequently, this is not conclusive to detect a specific GMO event.

3) To detect a particular event, *i.e*. the junction sequences in the integration site (plant-construct junction fragment) can be used to detect a specific transformation event. When the GMO is the result of a non-homologous recombination, the integration site is unique. When the same gene construct is used to produce different GMOs, this will be the only strategy to distinguish between GMOs containing the same gene construct. However, prerequisite for the development of such methods is the sequence information of the GMO as well as the availability of suitable reference material.

The relevance of a general screening PCR based on amplification of fragments common to several GMOs is highly dependent on the sought after objective and it cannot be used as a general method in all agricultural products as some GMOs do not have the general element in the construction. Therefore, the scope of using the general screening PCR shall be clearly defined. The general screening PCR may suggest the presence of GMO, which then need to be identified.

As described earlier, general screening PCR methods are methods that make use of genetic elements commonly used in GMOs (*e.g*. promoters and terminators). However, all PCR methods based on internal insertion sequences (including construct specific sequences) are suspected to be present in other GMOs and should be considered as screening methods. Thus screening PCR results in a positive or negative list.

In addition, general screening for the widely used 35S promoter or *nos*-terminator will not discriminate between the elements occurring naturally in infected plants or their presence in genetic constructs of GMOs. Especially in the case of rape seed and other *Brassica* members, a positive result from a 35S promoter screening may well be a false positive since these plants can be infected in nature by the cauliflower mosaic virus. However, by performing a CaMV-specific PCR based on genes normally not present in GMOs, false positives, as a result of virus infected plants, can be eliminated.

Identification of a particular GMO variety should be based on an unambiguous unequivocal signature. Because insertion of genes into plant genomes with the current technology is a random process, the border fragments (junction between the genome of the plant and the insert) is specific to each GMO and is the only approach to allow the unequivocal identification of each transformation event and its quantification.

Since the border fragments required for the identification and quantification of GMOs are at the moment not generally known, an intermediate situation has to be taken into account. As GMO approvals granted by the EU currently stand, certain

sequences internal to the insert (construct-specific) can be used for identifying and quantifying the EU-approved varieties (MacCormick *et al*. 1998). To detect non-EU-approved GMOs, the situation is even more complicated. This is described below.

A typical routine sequential test scheme for GMO detection is to initially screen samples for species-specific DNA (*i.e*. housekeeping genes as *e.g*. lectin gene (soybean samples) or invertase gene (maize samples) to determine whether DNA from that species can be detected. If DNA is detectable, samples are then screened using the general genetic elements, which detect multiple varieties of GMO-DNA. Positive results from this initial screening are further confirmed using tests, which screen for the specific genes or constructs used in the most common GMO crops. The exact tests used depends on the sample in question (*e.g*. Cry genes, EPSPS gene, Pat gene), or, more ideally, for the plant-construct junction fragments. This three-step process insures that any results reported have been confirmed using multiple screening systems. In addition, in the case of a positive GMO content, it is important to quantify the amount, in particular for the EU-approved GMOs in order to test for compliance with the 1% (0.5; 0,3 or 0.1 %) threshold level (see section 3.2.1.3).

3.2.1.2 PCR identification of non-EU-approved GMOs

As mentioned earlier, many of the GMOs contain the same genetic insert (= gene construct), which means that identification of a certain gene construct does not necessarily lead to identification of a specific GMO. There is a need from the inspection authorities for having a classification system that can link the identified sequences uniquely to a specific (approved) GMO. However, it is also important to realise that presence of GMO material may come from contamination of conventional fields from neighbouring fields. The chance that such contaminating GMO material belongs to the known and registered GMOs is high, but in theory it may also come from field trials with new types of GMOs, not yet approved for marketing. Identification of such GMO impurities poses even greater difficulties.

For identification of non-EU-approved GMOs, there are two situations at present:

1) Sequence data (and reference material) available. Event specific detection systems for these GMOs could be included within both the qualitative and quantitative tests.

2) No sequence data (and reference material) available. For this case the following strategies may be followed:
   Indirect method: In this case a subtractive approach could be implemented. Samples are tested for elements commonly used in GMOs (screening) and for the presence of approved GMOs and known non-approved GMOs with event specific tests. In case the event specific tests are negative and a positive test is found with one of the commonly used elements (screening) this could be considered as an indirect evidence for the presence of a non-known GMO.

<u>Direct method:</u> Another alternative to detect the non-approved GMOs could be a fingerprinting (or anchored PCR) method. It consists of amplification of the DNA of seed lots by using random primers in combination with a GMO screening primer (*e.g*. 35S, *nos*, *npt*II); the fingerprints thus generated could be able to differentiate non-approved GMOs from approved ones. This strategy can be considered as a direct way for the detection of non-approved GMOs.

The European Commission Joint Research Centre, Food Product Unit, ISPRA, Italy is at present working on elaboration of a molecular register, which will contain information on the molecular make-up of the different GMOs. Such a register provides the tools for the design of appropriate identification methods. However, the actual sequences are patented and in some cases unavailable to the public. Thus the elaboration of such a register requires agreements with the different companies that produce the GMO varieties. Some GMO analysing companies as *e.g*. Agrogene (Appendix 4 and 5) have confidential agreements with the GMO-producing companies, thus offering specific tests. These companies do not make their analysis methods (primer sequences) available to the public.

3.2.1.3 Quantitative PCR

The typical approach to quantification that is carried out in GMO analytical laboratories is to quantify based on analysis using one or more broad-spectrum primer sets that recognise common transgenic elements, such as the CaMV 35S promoter, the nos terminator, or one of the inserted genes. However, since different transgenic events contain these common sequence elements in different numbers, accurate determination of percent GMO cannot be achieved based on analysis of these common sequence elements. Different maize events may contain from 1 to 4 copies of the 35S promoter, and quantification based on this sequence can thus overestimate the percent of GMO in the sample.

Therefore quantification based on event-specific primers provides not only more precise results regarding the type of GMO present but also more accurate quantitative results.

<u>Quantification using conventional PCR:</u>
Conventional PCR measures the products of the PCR reaction at a single point in the reaction profile. Quantification can thus be achieved from “end-point” quantitative PCR (Peccoud, J. & Jacob, 1999). Because the relationship between DNA concentration and PCR signal is not linear, and because this relationship is not constant from one analytical run to another, the precision for quantification using conventional PCR is limited. Thus, the conventional PCR methods are semi-quantitative, while the Real-time PCR method (see below) is regarded as quantitative.

Another possibility for quantification using conventional PCR is competitive PCR (Figure 4). In a qualitative PCR reaction, an amplified product is synthesised from one primer pair. However, in a competitive PCR reaction, a second DNA fragment with known concentration is added to the reaction mixture. That second fragment, known as the competitor, has the same binding sites for the same primer pair but is different in size. By running a series of experiments with varying amounts of the synthetic DNA, it is possible to determine the initial amount of template DNA. If

the resulting PCR products are of equal intensity as detected by gel electrophoresis, then the initial amount of template DNA equals the initial amount of synthetic DNA (Figure 4).

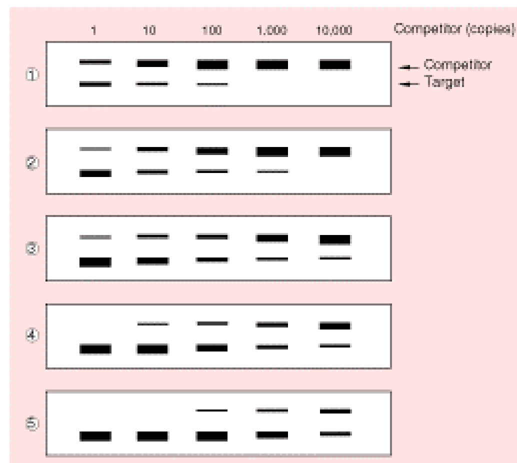

Figure 4. Schematic presentation of the principle of competitive PCR.

In a double-competitive PCR (DC-PCR) this principle is applied twice: once for a general housekeeping gene and its competitor and once for a GMO specific gene and its competitor. The results from both PCR reactions allow for the calculation of the GMO taken the total amount of the organism where the GMO has been derived from into consideration.

The competitive as well as the double-competitive systems have the distinct advantage that no additional equipment has to be acquired by the laboratories, if they are already performing qualitative PCR methods, since it is based on the same equipment. The competitive and the double-competitive PCR methods are semi-quantitative because they require a standard to be compared to. In this case, the standard is the known amount of synthetic DNA added. Consequently, the results can only indicate a value below, equal to or above a defined standard concentration.

It is expected that Real-time PCR gradually will replace these competitive PCR systems for quantification.

<u>Quantification using Real-time PCR:</u>

Real-time PCR is a system based on the continuous monitoring of PCR products. This is done via fluorometric measurement of an internal probe during the reaction. This probe consists of a short DNA fragment, which contains a fluorophor and a quencher (Figure 5). Due to their closeness, the quencher suppresses all the fluorescence of the irradiated fluorophor. The DNA sequence of the probe is designed to anneal exactly in the area to be amplified. In each PCR cycle, the DNA is first denatured to separate the two strands. The second step consists of the annealing of the primers and the internal probe and in the last step the DNA polymerase enzyme duplicates the DNA. The polymerases chosen for the real-time PCR show a strong 5'-3' exonuclease activity, which cleaves the internal probe into the nucleotides. Subsequently, the quencher and the fluorophor are not any longer in proximity and the fluorescence signal increases (Figure 5).

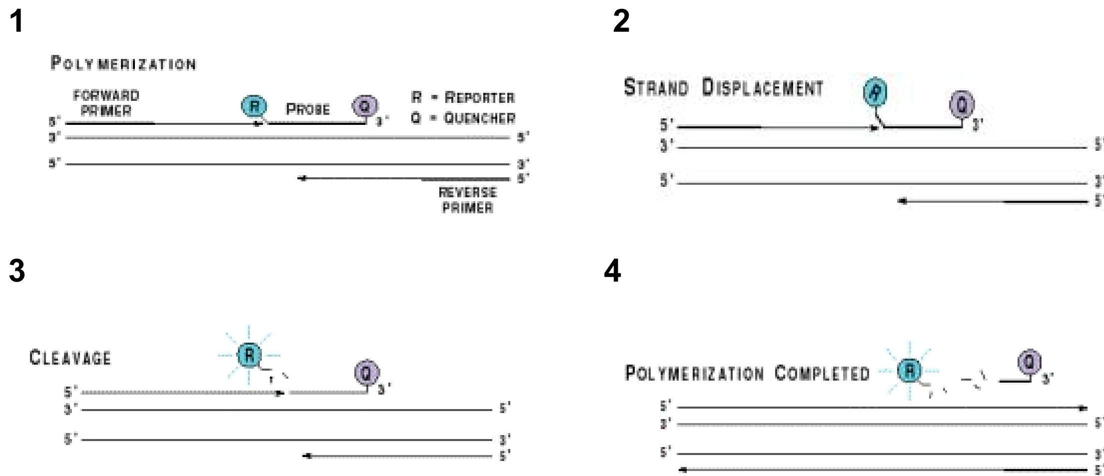

Figure 5. Principle of Real-time PCR.

It has been shown empirically that the concentration of DNA in the real-time PCR reaction is proportional to PCR cycle number during the exponential phase of the PCR reaction. Therefore, if the number of cycles can be determined that it takes for a sample to reach the same point in its exponential growth curve, it is possible to calculate the precise content of genetically modified DNA (Figure 6). The Real-time PCR method makes use of these principles to provide precise quantification of the GMO content of agricultural products. Each series of analyses includes the analysis of a full set of standards, giving rise to a standard curve. The results obtained for individual unknown samples are compared to the standard curve to determine the GMO content of those unknowns (Figure 6). Most real-time systems of instrumentation automate this analytical procedure.

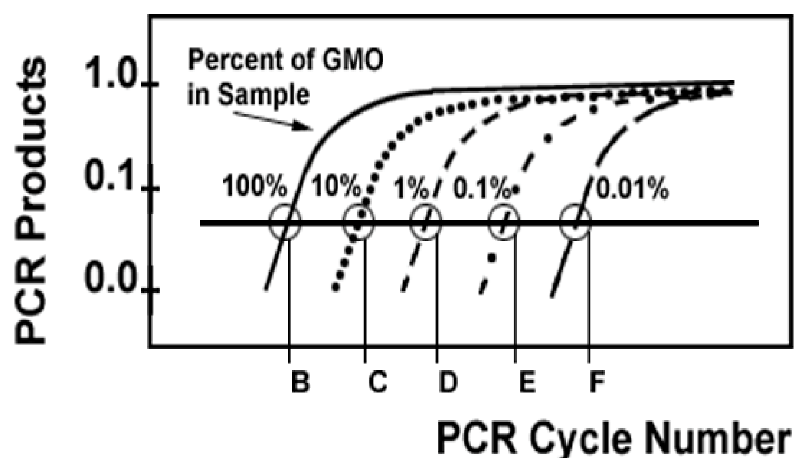

Figure 6. Timecourse of Real-time PCR (Log-Linear Co-ordinates).

The Real-time principles are currently commercialised by a number of companies and have been shown to be very precise. However, some of the disadvantages are the price and the training required. Therefore, only a small number of experienced laboratories have this instrumentation operational to date *i.e.* the method may be too expensive at present for routine screening programmes.

3.2.1.4 PCR detection levels

When considering a laboratory for GMO detection service it is important to ensure that the laboratory carries out appropriate controls, *e.g*. using housekeeping genes, contamination tests for CaMV, testing for false positive and false negative etc.

The PCR reaction itself can be used to verify the integrity and quality of the extracted DNA. The principle of this method consists in performing PCR on a target that is either universal (processed products) or plant species-specific (raw materials as *e.g.* seeds) and then to relate the obtained quantity of amplicons to the initial quantity of total DNA. This can be done either by end-point (conventional) PCR or Real-time PCR.

Limits of detection.

In general, PCR based methods have a threshold detection of 0.01 %. This limit is caused by the amount of DNA that is introduced into the reaction. If a sample of maize genomic DNA contains 0.01% GMO material, the number of GMO targets in a PCR reaction will on average be very small (one to four target molecules). The total number of maize genomes in each PCR reaction causes the reason for this.

The size of the maize genome is about $4.5X10^9$ base pairs and the amount of sample DNA introduced into PCR reactions is normally around 25 to 75 ng (sometimes up to 200 ng). Using Avogadro's number ($6.023X10^{23}$), one can calculate from these values that the actual number of maize genomes present in a PCR reaction will range from around 10.000 (50 ng sample DNA) to around 40.000 (200 ng sample DNA). If the concentration of GMO genomes in the sample is 0.01% (=1 in 10.000) the number of GMO genomes (the number of GMO target molecules) would be 1 for a 50 ng DNA sample to 4 for a 200 ng DNA sample. Because the soybean genome is smaller than that of maize ($2.5X10^9$ base pairs), sampling statistics are a more favorable little better for soybeans than for maize.

Limits of quantification.

Even though 0.01% is the limit of detection using PCR, quantitative analysis is not possible in this concentration range. In samples from a DNA preparation whose actual composition is 0.01% GMO material, the number of GMO targets in any given sample could be zero, one, two, three, four or more. Amplifying these samples will lead to results having substantial differences in signal intensity. These differences will not be related to the actual GMO content of the original sample but will be due to the statistical variations related to sampling of that DNA preparation. Thus, differences in signal intensity cannot be correlated with quantitative differences in GMO content in samples that contain low GMO levels such as 0.01%. Therefore, most laboratories set the limit of quantification ten-fold higher at 0.1% to avoid the problems with precision that occur near the limit of detection.

Furthermore, to reliably quantify at the 0.1% level (1 in 1.000), thorough statistics require that at least 10.000 seeds should be homogenised and thoroughly mixed, and duplicate samples of this homogeneous powder subjected to DNA analysis. Several companies have shown that it is a practical reality to operate at this threshold.

3.2.2 Microarrays

Microarray technology (DNA chip-technology) has been developed in recent years for automated rapid screening of gene expression and sequence variation of large number of samples. Microarray technology is based on the classical DNA hybridisation principle, with the main difference that many (up to thousands of) specific probes are attached to a solid surface (Figure 7). Different formats are known, *e.g.* macroarrays, microarrays (Figure 7), high-density oligonucleotide arrays (gene chips or DNA chips) and micro-electronic arrays (Freeman *et al*. 2000). In DNA chips, short oligonucleotides are synthesised onto a solid support, whereas in DNA arrays, PCR products, corresponding to either genomic DNA or cDNA sequences, are deposited onto solid glass slides (microarray) or nylon membranes (macroarray). Micro-electronic arrays consist of sets of electrodes (capable of generating a current) covered by a thin layer of agarose coupled with an affinity moiety.

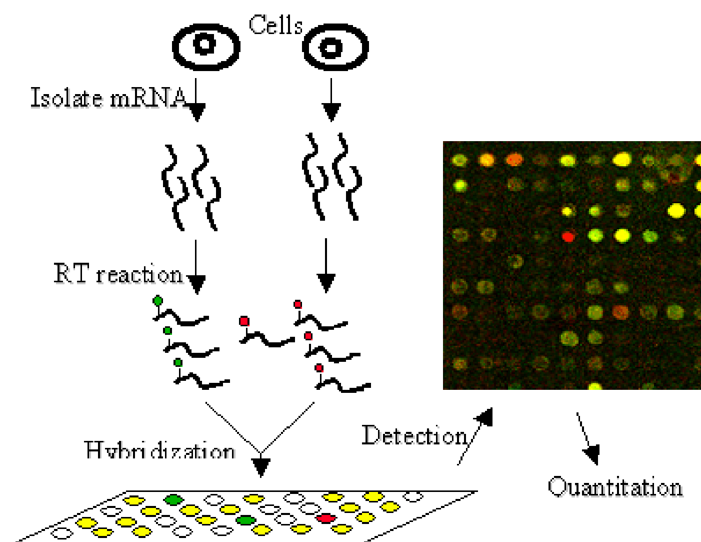

Figure 7. Flowchart of the microarray principle.

These techniques are developing rapidly and have many advantages but also some limitations, at least at present. Since the techniques are very sensitive and still under development, they are limited to expert laboratories. Very recently, the first GMO chip kit developed by GeneScan Europe (distributed by Scil Diagnostics) (Appendix 4 and 5) has been introduced to the market. The new DNA chip screens and identifies GMOs in raw materials, processed food, and animal feed. The GMO chip kit combines previously separately performed screening and identification

procedures in a single test. With additional tests for plant species, the chip provides results of a total of 14 separate analyses. The GMO chip kit package contains all reagents as well as analytical software (Signalyse®). The chip analysis will also be introduced as a service for the customers of GeneScan-Europe's GMO service laboratories.

The present GMO chip kit detects species-specific DNA of plants and viruses, generally used genetic construction elements, and specifically introduced genetic modifications for the identification of approved and non-approved plant varieties. The GMOchip version "The European" detects specific DNA from soybean, maize, oilseed rape, rice, CaMV (species) and the following GMOs: RR-soybean, Maximizer Bt 176 maize, Bt11 maize, Yieldgard Mon810 maize and Bt-Xtra maize (see Appendix 3). Further versions of GMO chips adapted to regulations in regions and countries other than the European Union are under development. In addition, GMOchip allows screening for all GMOs with the CaMV 35S promoter, Nos-terminator, bar-gene and pat-gene. However, proper quantification is currently problematic with microarrays, this is described later.

GeneScan-Europe in combination with Clinical Micro Sensors (a division of Motorola based in Pasadena, California) is developing a micro-electronic array, which they call "eSensor". The "eSensor™" is a small circuit board laced with up to 36 gold electrodes. Each of these is linked to more than a billion identical single-stranded DNA molecules, and each DNA strand is attached to a kind of electrically conductive carbon compound known as a ferrocene molecule. The DNA molecule at an electrode corresponds to particular fragments of DNA found in genetically modified crops. When a strand of DNA comes into contact with its complementary target, the two bind together. This reaction holds the ferrocene molecule close to the electrode's surface, where it changes the current passing through an electrode. A device measuring this current can then be used to gauge what kind of DNA has been detected, and thereby estimate how much of it is present in the sample.

The device is already available for use in the laboratory but still needs more work before it will function effectively in the field. Currently, a sample must undergo a lengthy chemical preparation before testing. Its DNA has to be extracted and then replicated so that the "eSensor" may detect it more easily. Clinical Micro Sensors works on replacing this step with a microfluidic system, which will prepare the sample and replicate the DNA automatically within an hour.

The next step will be to couple the system with a detector in a hand-held device that can be used "truck side" *i.e.* as the food leaves the field after harvesting. Such devices ought to be able to detect DNA present in as little as 0.025% of a sample. Since "eSensors" can detect many types of DNA simultaneously, a sample of grain or processed food only needs to be tested once in order to screen for several dozen different kinds of genetic modification.

As microrray technology has expanded, quantitative comparison of data within and across microarray platforms has proven difficult. The main reasons for this are a lack of universal references and the variety of data analysis methods in use. Novel approaches for data normalisation and statistical interpretation will improve standardisation and validation of microarray data providing accurate and precise data extraction that enables quantitative comparison of microarray data across experiments.

3.3 Comparison of the different methods

Herbicide bioassays are inexpensive and very accurate in identifying GMOs with the particular trait in samples of viable seed/grain. Testing individual seeds performs quantification of the GMO level, normally 400 seeds are tested per sample. The accuracy is dependent on the germination: the higher germination the higher is the confidence level of the test. Only viable seed or grain can be tested (no processed products), and each test requires seven days to complete. The potential error of accuracy increases as the germination level of the sample decrease. Furthermore, bioassays require separate tests for each trait in question and at present the tests will not detect non-herbicide tolerance traits. Therefore, the tests are only of limited value for inspection authorities.

ELISA is faster and less expensive than PCR based methods and can be set up in any laboratory. Several companies sell specific kits (Appendix 4), which are used by GMO testing laboratories. The low-technological lateral flow sticks are fast and give semi-quantitative results that assure there is not more than a given amount of GMO material in the sample. The flow sticks will not guarantee that no measurable amount of GMO material is present; for that, the more-sensitive micro-titer plate kits must be used. Nonetheless some companies claim that depending on the trait, the lateral flow sticks can detect levels down to 0.01%.

It is important to remember that ELISA and lateral flow sticks are trait-specific and thus cannot identify a GMO where several varieties may have the same trait incorporated. Therefore, the immunoassays in general can be considered as screening methods. Since the same target protein can be found in different GMOs, antibody-based assays may not be discriminating (for example, the maize varieties Bt-176, BtII and Mon810 contain the same Cry protein). Thus, at present, PCR based methods are the methods, which allow the most precise GMO identification and have the highest sensitivity, in terms of detection limits.

Furthermore, the immunological tests present some problems: they will tend not to work on denatured protein, and the specific type of protein or variety must be known and expressed at the sampling time. Furthermore, the foreign proteins may vary in expression levels in different plant tissues, making quantification difficult. Differences in the expression of the protein in different varieties of a species would influence the quantitative measurements, or at least calculations of the proportion of genetically modified material in the tested specimen, and must be taken into consideration. The sensitivity of immunoassays is slightly lower than PCR techniques. The detection limit for *e.g*. GM soybean is 0.1% in 100% soybean flour. If the presence of non-EU-approved GMOs in conventional seed lots is below 0.1%, false-negative results can be obtained. For PCR approaches a detection limit of 0.01 % is described (section 3.2.1.4).

The DNA microarray technology offers the same advantages as PCR as the method allows the most precise GMO identification (similar problems as in PCR based methods regarding target sequences). Furthermore, the advantage is that screening and identification is carried out in a single step in contrast to the PCR based approaches. The microarray, in principle, enables the detection, identification, and quantification of large numbers of GMO varieties present in a sample in one single assay. Furthermore, microarrays are very flexible, as new varieties can be included in the screening procedure by adding additional sequences to the array.

However, due to the problems with comparison of data within and across microarray platforms, *e.g*. that the current available linear labelling of the DNA does not give the required increase in fluorescence signal, the microarray technology is still used as a first-line screening in a two-tiered approach. A second step will still be necessary to more exactly quantify the amount of detected GMO varieties.

4. Validation and standardisation of the analytical methods

The request for powerful analytical methods for routine detection of GMOs by accredited laboratories has called attention to international validation and preparation of official and non-commercial guidelines. Among these guidelines are preparation of <u>c</u>ertified <u>r</u>eference <u>m</u>aterial (CRM) (see below), sampling, treatment of samples, production of stringent analytical protocols, and extensive ring-trials for determination of the efficacy of selected GMO detection procedures.

Validation of methods is the process of showing that the combined procedures of sample extraction, preparation, and analysis will yield acceptably accurate and reproducible results for a given analysis in a specified matrix. For validation of an analytical method, the testing objective must be defined and performance characteristics must be demonstrated. Performance characteristics include accuracy, extraction efficiency, precision, reproducibility, sensitivity, specificity, and robustness. The use of validated methods is important to assure acceptance of results produced by analytical laboratories. According to European Union legislation state laboratories participating in inspection should, whenever possible, use validated analytical methods. This is also the case for all laboratories aiming at accreditation.

Each new method should be tested in ring- trials using numerous laboratories in order to demonstrate reproducible, sensitive and specific results. In these ring-trials the same measurements should be assessed on identical materials. The experimental designs of each trial are crucial and several questions should be considered when planning such experiments. Examples of important issues to consider include availability of satisfactory standards, number of laboratories and how they should be recruited. It is also necessary to specify the manner of calculating and expressing test result.

No single validated method has yet been developed which is capable of accurately determining all GM products in a timely and cost effective manner. Testing programs will need to incorporate the best qualities of each technology in developing testing programs. The collaborative efforts of many organizations will be required to facilitate the development of reliable, validated diagnostic tests with broad global acceptance among users and regulators.

Methods based on a relatively expensive instrumentation, requiring substantial efforts in training and available only to a limited number of participants as *e.g*. Real-time PCR or Microarrays for validation studies may not be useful at the moment, as methods to be implemented in routine laboratories on an European scale.

Some examples of methods that has been validated or accredited recently are given below:

A standardised method to identify Roundup ReadyTM soybean (Zagon *et al*. 1998), ring trials for the quantitative measurement of Roundup ReadyTM soybean? content as well as Bt176 maize using real time quantitative PCR have been accomplished by the BgVV (Federal Institute for Health protection of Consumers and Veterinary Medicine in Germany). In addition, qualitative PCR-based methods to identify Bt176, Bt11, T25 and MON810 maize have been validated by the BgVV.

A PCR and an ELISA method for Roundup ReadyTM soybean and a PCR for Maximizer maize (Bt176) have been validated for commercial testing of grain by the European Union's Joint Research Centre, JRC (Lipp *et al*. 1999; 2000). An ELISA for MON810 maize has also been validated and is now being issued by AACC (American Association of Cereal Chemists) (Stave *et al*. 2000). A prenormative standard is set for a protein method (published as draft European standard, April 2001), which in the annex introduces the ELISA for detection and quantification of the RoundUp Ready trait in soy flour.

The so-called Varietal ID PCR methods (based on primers that span unique sequence junctions) developed by Genetic ID (Appendix 4) has been accredited through the United Kingdom Accreditation System (UKAS).

Ideally, GMO-testing laboratories should participate in an internationally recognised external quality control assessment and accreditation scheme. In accordance with this, authorised laboratories (approved for official inspection purposes) must participate regularly in appropriate proficiency testing schemes.

4.1 Certified Reference Materials for GMO Detection

Reference material is material with sufficiently stable and homogeneous properties and well established to be used for calibration, the assessment of a measurement method or for assigning values to materials. Certified reference material (CRM) is reference material accompanied by a certificate issued by a recognised body indicating the value of one or more properties and their uncertainty. The certified values of these materials have been established during the course of a certification campaign including interlaboratory studies (which should be available upon request). In the absence of CRM, standards validated by a laboratory can be used.

For compliance with the 1% threshold level, certified reference materials for precise quantification and method validation are needed. According to commonly accepted rules, the production of reference materials should preferably follow metrological principles and should be traceable to the SI system. Arbitrary definition of measurement units could lead, as a consequence, to difficulties with non-consistent standards and a lack of long-term reproducibility. In the future, efforts should be concentrated on establishing reliable quantification methods accompanied by the production of reference materials with high DNA quality and DNA degraded under controlled conditions (simulating real samples in food production) using very well characterized base materials.

Protein reference materials are critical for the validation of externally operated immunochemistry processes. Reference materials can be derived from a number of

production sources, and can take on a variety of final forms (stabilized plant extracts to highly pure protein).

Three types of certified GMO reference samples for GMO testing are especially needed: DNA-CRM, matrix-CRM for events of major importance, and protein-CRM. An important issue to consider is that the CRMs are stable and non-degraded. Often problems with degradation of CRMs are encountered.
The European Network of GMO Laboratories has prepared a list of wishes concerning CRMs for GMO inspection as follows:

For production of GMO-CRMs one variety per transformation event common in USA and EU should be used. GMO and non-GMO should be corresponding near-isogenic lines. For each EU-approved GMO varieties CRMs are needed ((Bt176, Bt11, T25, Mon810, Mon809, RR soy, Ms1/Rf1, Ms3/Rs8, topas 19/2 and probably soon for T25+Mon810, GA-21). CRMs for some special cases of US-approved lines like CBH351 CRMs should be available. Powdery reference materials from certified commercial seeds for relative quantification of GMO should be available with a GMO content of 100, 5, 2, 1, 0.1, and 0 %. Plasmids would be helpful for absolute quantification (native and competitors sequence, transgenic GMO – preferably including both edge fragments – and housekeeping sequences).

European Union's Joint Research Centre, Institute for Reference Materials and Measurements, Geels, Belgium, is currently developing a system for distribution of GMO reference material. Website: http://www.irmm.jrc.be/.

5. Conclusions and future aspects

As described in this report, the detection, identification and quantification of the GMO content in seed samples and in food or feed products is a great challenge for the inspection authorities. The existing analytical methods for GMO testing leave the inspection authorities with many choices and compromises. The choice of method depends on why the information is needed. In general, the more demanding the consumers, the more expensive the cost of providing the information will be. Also, the more exact the procedure, the more expensive and time consuming it will be. Furthermore, it is obvious that international collaboration is needed to ensure that the methods offered by the different companies holds promises. This can be done by elaboration of:

- Further research to understand the appropriateness of DNA and protein based methodologies
- Compatibility between methods
- Appropriate protocols for validation studies and for proficiency testing
- Make appropriate reference materials readily available.

Methods, which can guarantee absence of non-approved GMOs in seed samples – even at the suggested 0.1% level – does not exist at present. The chance of detecting non-approved GMOs would be significantly improved if the American companies that produce the GMOs would describe the constructions and make them accessible to the National governmental bodies (inspection authorities).

In addition, fundamental problems with the existing screening methods exist: PCR is the most widely applied method and often an initial screening for GMO is carried out using the well-known 35S and NOS-promoters as targets. This screening will not reveal the presence of GMOs with other promoters. In addition, the 35S test does not work reliably in *e.g*. Brassicas, as natural infection with the CaMV is common, blurring the results. In the future, it is likely that all kinds of promoters will be used. Research is going in the direction to develop GM plants with inducible promoters that activate specific traits when needed. One of the major challenges for the future development of analytical identification methods will be to develop methods that facilitate screening for all the promoters used worldwide.

Furthermore, the number of different GMOs is expected to grow and thus it is necessary to currently revise and update the analysis methods. In the first generation of GM plants relatively few different genes - encoding herbicide tolerance and insect resistance - have been used. The second generation of GM plants includes a larger variation of genes and traits. According to Kjellsson & Strandberg (2001) different new traits include:

- Increased stress tolerance (*e.g.* drought, frost, and salt)
- Changed growth characteristics (*e.g*. male sterility systems, fruit ripening phenology, increased growth rate, and nitrogen fixation)
- Changed chemical composition (*e.g.* proteins, oil content, starch, and vitamins)
- Production of pharmaceuticals (*e.g*. vaccines, hormones, and enzymes)
- Production of industrial compounds (*e.g*. biomass, lignin, and plastics)
- Bioremediation (decontamination of toxic soils).

There is a tendency that the number of different traits inserted into the same plant increases. Many of the internationally approved varieties already have more than one trait inserted (Appendix 3).

It can be expected that detection of GMOs will become more complicated in the near future. Many other GM varieties, besides the few that have a market approval in the EU, are about to be approved or are already on the market in important trading partners of the EU. For the maintenance of European regulatory requirements it will therefore be necessary to develop or implement more powerful screening and detection methods soon. The most prominent example of an innovative and promising approach is the application of the microarray technology for GMO detection. Among the advantages is the flexibility of the DNA microarray technology as new GMO varieties can be included in the screening procedure simply by adding additional sequences to the array. Still the availability of the sequences is crucial, but there are suggestions, which demand that the GMO-producing companies release sequences along with the proposal for approval of new GM varieties.

6. References:

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