Report

of the

CORESTA Task Force

Genetically Modified Tobacco:

Detection Methods
CONTENTS

List of Contributing Authors........................................................................................................ 3
   Summary.................................................................................................................................. 4
I.  Introduction .......................................................................................................................... 6
II.  Sampling Strategy and Methods......................................................................................... 8
III. Sample Preparation and DNA Isolation Methods ............................................................ 16
IV.  PCR-Based Screening Methods ....................................................................................... 22
V.  Semi-Quantitative and Quantitative PCR Methods......................................................... 42
VI.  Immunological Detection Methods .................................................................................. 59
VII. Selectable Genetic Marker-Based Detection Methods.................................................... 65
VIII. Official and Validated Methods ....................................................................................... 73

APPENDICES

I.  Internet Databases on GM Tobacco Field Trials............................................................... 88
II. Tobacco DNA Extraction for PCR Analysis........................................................................ 89
III. Chinese Method for Screening GM Tobacco....................................................................... 93
IV.  Glossary of Terms ............................................................................................................ 114
## LIST OF CONTRIBUTING AUTHORS

<table>
<thead>
<tr>
<th>Author</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mr. Gregor Bindler</td>
<td><em>Philip Morris International, Switzerland</em></td>
</tr>
<tr>
<td>Dr. François Dorlhac de Borne</td>
<td><em>SEITA, France</em></td>
</tr>
<tr>
<td>Dr. Ferruccio Gadani</td>
<td><em>Philip Morris International, Switzerland</em></td>
</tr>
<tr>
<td></td>
<td><strong>Task Force Coordinator and Editor</strong></td>
</tr>
<tr>
<td>Dr. Evan Gregg</td>
<td><em>British American Tobacco, UK</em></td>
</tr>
<tr>
<td>Mr. Zhaokui Guo</td>
<td><em>Heilongjiang Tobacco Research Inst., P.R.China</em></td>
</tr>
<tr>
<td>Dr. Hubert Klus</td>
<td><em>Austria Tabak AG, Austria</em></td>
</tr>
<tr>
<td>Dr. Martin Maunder</td>
<td><em>Advanced Technologies (Cambridge) Ltd, UK</em></td>
</tr>
<tr>
<td></td>
<td><strong>Co-Editor</strong></td>
</tr>
<tr>
<td>Dr. Lutz Mueller</td>
<td><em>R. J. Reynolds Tobacco GmbH, Germany</em></td>
</tr>
<tr>
<td>Dr. Hideaki Negishi</td>
<td><em>Japan Tobacco Inc., Japan</em></td>
</tr>
<tr>
<td>Mr. Hans Pijnenburg</td>
<td><em>Philip Morris International, Switzerland</em></td>
</tr>
<tr>
<td>Dr. Martin Ward</td>
<td><em>Advanced Technologies (Cambridge) Ltd, UK</em></td>
</tr>
<tr>
<td></td>
<td><strong>Task Force Secretary and Editor</strong></td>
</tr>
<tr>
<td>Dr. Jacques Zuber</td>
<td><em>Philip Morris International, Switzerland</em></td>
</tr>
</tbody>
</table>
SUMMARY

The advent of modern biotechnology and the accelerated pace of scientific discovery in the areas of genetic modification (GM) and genomics are introducing fruitful developments in both the medical and agricultural sciences. However, the presence of genetically modified crops in food products has recently become a widely debated and contentious issue in Europe and other regions. Despite the substantial growth of the global GM crop area to 40 million hectares (as of 1999), public concern is being increasingly raised with regard to the potential impact of the technology. To respond to consumer preferences, Identity Preservation (IP) programs that can ensure traceability and segregation of GM crops are gradually being implemented for certain sectors of commodity crops (e.g., soybean and corn). Moreover, in many countries legislation has been introduced that mandates the labeling of food which contains or has been produced from a genetically modified organisms (GMO). Reliable tests are therefore being developed to identify transgenic materials in food by the detection of DNA (deoxyribonucleic acid) and proteins.

Although applications of biotechnology to tobacco and other Nicotiana species have been limited to the use of this plant as a model system and the evaluation of traits in field experiments, there are examples where new GM tobacco varieties have been developed and are awaiting approval before commercialization. Analytical methods that can detect genetic modification in tobacco are being developed in several laboratories.

During 1998, the CORESTA Scientific Commission has held discussions on this subject and recommended the creation of the Task Force Genetically Modified Tobacco - Detection Methods, which was officially inaugurated in December 1998 with the following mandate (quoted in the original wording):

- Review methods for detecting genetically modified tobacco (including cured leaf, tobacco products, plants and seed);
- Propose principles for sampling procedures.

Accordingly, the Task Force has thoroughly investigated these issues and has prepared a report, which reviews the recent scientific literature in the area of GM crop analysis. Eight Sections and three appendices provide details on the scientific and technical aspects of methodologies that can detect the presence of introduced DNA or novel proteins in a tobacco plant, and on sampling principles.

After the Introduction (Section I), the review starts with Section II on Sampling Strategies and Methods, which summarizes the main steps of a sampling plan and includes a brief glossary of terms that are frequently used in describing sampling procedures. ISO standards and practical approaches with potential relevance to GMO analysis are examined. Moreover, this section emphasizes the difficulties in devising a suitable sampling strategy until acceptance criteria (de minimis thresholds) have been defined and established for tobacco.

Section III, Sample Preparation and DNA Isolation Methods, summarizes the main strategies for the isolation and purification of plant genomic DNA suitable for the polymerase chain reaction (PCR), and highlights the importance of selecting the appropriate protocol for materials such as cured tobacco leaf.

Section IV, PCR-Based Screening Methods, reviews PCR methods to detect unique fragments of DNA that are very widely used in plant genetic modification. These fragments such as the 35S promoter from cauliflower mosaic virus, and the nos promoter and nos terminator from Agrobacterium tumefaciens are therefore diagnostic of genetic modification.
The advantages and drawbacks of confirmatory methods (e.g., nested PCR, nucleotide sequencing) are also highlighted.

The key issues of method validation (between different laboratories) and availability of GM tobacco reference materials, which are also discussed in Section VIII, are formulated here.

Section V on Semi-Quantitative and Quantitative PCR Methods includes a mathematical evaluation of PCR product formation, which shows the limits of conventional PCR in quantitative assays. The main approaches for semi-quantitative and quantitative determination of GMO levels, i.e. the quantitative competitive PCR (QC-PCR) and the real-time PCR, respectively, are reviewed and analyzed in detail.

Section VI, Immunological Detection Methods, provides insight into testing methods required to detect and quantify specific novel proteins in GM plant varieties, with a focus on adaptations of the widely used immunological assay ELISA (enzyme-linked immunosorbent assay), which is reported to be less sensitive than PCR.

Section VII, Selectable Genetic Marker-Based Detection Methods, discusses the use of GM crop analytical strategies based upon identifying the presence of selectable marker genes in transgenic plants. The activity of marker genes such as the neomycin phosphotransferase II (NptII) can be detected in seeds or plantlets directly (e.g. assessing the impact of a selective agent in seedling tests), or indirectly (e.g. enzymatic assay, bacterial complementation). However, at present these methods are less sensitive than PCR and are difficult to apply to cured leaf.

In Section VIII, Official and Validated Methods, the Task Force sets out to examine an additional dimension of the GMO analysis technical issues, that of international method validation and standardization. The first part of the section reviews the current status of official and validated methods in Switzerland, Germany and the European Union. The second part summarizes the activities of working groups within national and international bodies such as the French Standardization Association (AFNOR) and the European Committee for Standardization (CEN), that have recently acknowledged the need for harmonized protocols to validate GMO analysis methods. A critical issue appears to be the lack of a central repository with both information on GMOs (e.g., sequence data, primers, proteins) and samples (e.g., DNA, proteins, antibodies) of conventional and GMO reference materials.

The three Appendices include information from practical laboratory experiments conducted at laboratories of Task Force members, and a list of internet addresses of public databases featuring information on GM plant field trials.

Finally, a Glossary of Terms has been included to facilitate the reading of the Report by those readers who may not be familiar with genetic modification of plants and GMO analysis techniques.
I. INTRODUCTION

As modern crop biotechnology is coming of age, the commercial cultivation of genetically modified (GM) plant varieties has grown to 40 million hectares distributed in twelve countries [1], which represents approximately 4% of the total world acreage. Most current GM varieties feature enhanced agronomic (or input) traits, such as built-in protection from pest and disease, that can reduce production costs and provide increased flexibility in crop management. More recently, genetic modification has been successful in enhancing quality (or output) traits of crop plants, e.g. modified fatty acid or amino acid profiles in vegetable oils and cereals, respectively.

However, the presence of genetically modified organisms (GMOs) in food products is becoming increasingly controversial in Europe because of public concern with regard to the potential impact of GMOs on the health and the environment. To respond to consumer preferences, the biotechnology, trade and food industry are gradually implementing Identity Preservation (IP) programs that can ensure traceability and segregation of GMOs in certain sectors of commodity crops, such as soybean and corn. Moreover, recent EU regulations have introduced GMO labeling provisions that require verification of the GM or non-GM status by the use of methods for the analysis of DNA (deoxyribonucleic acid) and proteins [2]. This has led most official government and several industry laboratories to develop analytical methods that can assist in the enforcement of the labeling provisions and in the implementation of IP programs by tracing GM agricultural materials throughout the entire supply chain, from seed to shelf.

Tobacco (Nicotiana tabacum L.) has been the most widely used model system for investigations in the areas of plant physiology, biochemistry, molecular biology and genetic engineering, and was the first plant to be genetically modified in 1983 [for a review, see 3, 4 and 5]. Potential commercial applications of biotechnology to tobacco as a crop have been limited to field trials conducted in several countries (see Appendix I), although there are examples where new GM tobacco varieties have been developed and are awaiting approval before commercialization.

Given the importance of GMO analysis methods in the research, development and monitoring of GM tobacco varieties, CORESTA considered establishing a Task Force to review the scientific and technical aspects of methodologies that can detect the presence of newly introduced DNA or novel proteins in a tobacco plant. The Task Force Genetically Modified Tobacco - Detection Methods was therefore created within the Phytopathology Study Group of CORESTA in August 1998 and was officially inaugurated in December 1998 with 24 tobacco industry members from 13 countries.

This report is an overview of recent scientific literature in the area of GMO analysis and also includes information from practical laboratory experiments conducted at laboratories of Task Force members.
Note. The use of trade names and commercial sources throughout this report is for information purposes only and does not imply endorsement by CORESTA or CORESTA Task Force members. In addition, the views expressed in the document are those of the individual authors and may not necessarily reflect those of CORESTA.

References


II. SAMPLING STRATEGIES AND METHODS

Introduction

There are many reasons why one would want to sample tobacco or tobacco products for analysis. The determination of essential leaf tobacco constituents, such as nicotine, sugars and proteins, and of agrochemical residues are routine procedures. Frequently, samples are taken from batches to make smoking articles for sensory testing. The measurement of the “tar” and nicotine yields in cigarette smoke is probably the most important analytical practice in the Industry. Last but not least, the examination of the crop or of finished product for transgenic tobacco requires appropriate sampling.

This is to say that sampling procedures for GMO testing are quite comparable and similar to sampling in general and can, in principle, often be done in combination. Special attention, however, must be paid to certain aspects which are specific for GMO testing. Here, it is important to differentiate between the two principal reasons of all analytical determinations.

On the one hand, the purpose may be the assessment of chemical or physical characteristics, on the basis of means, for a certain quantity of material or product. These means may then be taken as absolute numbers (e.g., nicotine and sugar content of raw tobacco) or compared to existing targets (e.g., weight and diameter of cigarettes) or evaluated in reference to existing ceilings (e.g., maximum residue levels for agrochemicals on raw tobacco or in finished product; yields for “tar” and nicotine in cigarette smoke).

On the other hand, the determination of constituents or contaminations may be performed to demonstrate their absence (in practice, presence below the limit of quantitation) from the material or product under investigation. This is done in cases where, for instance, the maximum residue level for an agrochemical is set at zero. Similarly, under today’s conditions, it is reasonable to assume that most analyses for transgenic tobacco have the purpose of demonstrating its absence from the material of interest.

However, it must be emphasized that no sampling strategy can be devised that would allow one to guarantee the total absence of transgenic material. Until acceptance criteria (thresholds) are defined and established also for tobacco (products), devising a suitable sampling strategy will always be difficult.
General Principles of Sampling

Sampling is the well-planned collection of representative parts from a larger amount of material for the purpose of analyzing certain physical or chemical properties. It may be required for two reasons: Either the whole amount of material under investigation is so large that it cannot be subjected to the analytical procedure in its entirety, or the method of testing is destructive (which is the case with most chemical methods) and no intact material is left after the analysis for commercial use or any other purposes.

Good sampling is an essential requirement for valid analytical measurements. Practical experience has shown that, not infrequently, too little attention is given to proper sampling techniques in relation to the analytical methodology subsequently applied to the samples. In these situations, the quality of the analytical procedure may be (far) superior to the quality of sampling – without true value being added to the whole investigation.

The principal steps in sampling and execution of analyses are the following:

1. **Design of a sampling plan**
2. **Collection of samples from the totality of material under investigation**
3. **Reduction and preparation of laboratory samples**
4. **Performance of analysis of the samples**
5. **Evaluation and interpretation of analytical results**

The first three steps are discussed in this Chapter (the others being dealt with elsewhere). For this purpose, it may be helpful to explain a number of terms that are frequently used in describing sampling procedures. The following definitions are generally accepted and have been slightly modified and adapted for easier use in tobacco sampling:
### Consignment
the quantity of tobacco dispatched or received at one time and covered by a particular contract or shipping document. It may consist of one or several lots

### Lot (batch)
tobacco, presumed to be of uniform characteristics, taken from the consignment and available for analysis; a tobacco lot is generally from the same origin and variety, often specific for leaf position, color, ripeness, etc.

### Sampling unit
a unit of the lot which is packaged separately (bale, case, carton, basket, sack); for bulk tobacco, a sampling unit should be 100 kg

### Increment
a small quantity of tobacco taken from a single position in the sampling unit or the lot, generally by means of a sampling device; a series of increments is taken from different positions of the sampling unit or lot

### Basic sample
the quantity of tobacco obtained by combining and mixing the increments taken from a specific sampling unit or lot; if the basic sample is further subdivided into identical portions, these are called reduced samples

### Laboratory sample
a quantity of tobacco taken from the basic sample (or reduced sample) and intended for analysis; at this point, reference samples should routinely be set aside (e.g. for the seller, the buyer and for control analyses)

### Test sample
a sample prepared from the laboratory sample according to the test procedure specified in the test method

### ISO Sampling Standards with Relevance for GMO Testing

There are a number of official standards in place for the sampling of tobacco and tobacco products. It is worthwhile to examine them with respect to their utility in the present context.

The International Standard ISO 8243:1991 “Cigarettes - Sampling” was originally developed by ISO/TC 126 in 1988 and re-issued in 1991. It describes the sampling of finished product (cigarettes), essentially for the purpose of determining, and ranking, smoke yields for “tar” and nicotine. The standard contains plans for sampling both at the manufacturer’s (importer’s or wholesaler’s) location, with samples being taken at a certain point of time or over a given time period, and from the market.

In practice, however, the question of whether transgenic tobacco is contained in finished product at all or not should reasonably be examined, and controlled, at the level of the raw material, tobacco. Therefore, the sampling of cigarettes for GMO testing according to ISO 8243:1991 may be of limited practical importance.
The document which obviously has the highest practical relevance to the question under discussion is the International Standard ISO 4874:1981 “Tobacco – Sampling of batches of raw material – General principles”. It is currently under revision in the form of a first working draft (ISO/TC 126 N 563 of June 1997). The standard “specifies the general principles to be applied when sampling batches of raw tobacco in order to assess either the mean value of one or more of its characteristics, or the heterogeneity of one or more of its characteristics.” In general, heterogeneity describes the variability of a characteristic measured in different batches. Heterogeneity, of course, also exists if some analytical values are zero (indicating the absence of the analyte) and others are not zero (demonstrating its presence in certain amounts). Therefore, the standard does have relevance for sampling if the absence or presence of a certain characteristic (such as transgenic tobacco) is to be assessed.

The standard is applicable to the sampling of batches of various types of leaf tobacco as well as pre-treated raw tobacco. It provides a range of definitions, guidance for contractual arrangements, recommendations for sampling and sample storage equipment, and detailed rules for the sampling procedure (including the selection of sampling units, the number and kind of samples taken and the sampling report).

With GMO testing in mind, special attention should be given to the comment in Note 5 of ISO 4874:1981 on heterogeneity testing and the recommendations regarding the treatment of damaged sampling units (in Section 6.2 of ISO 4874:1981).

Turning the attention now to the other end (the beginning) of the production chain, there is no specific standard for the sampling of tobacco seeds. Practical guidance may be taken from a document developed by ISO/TC 34, namely the International Standard ISO 950:1979 “Cereals – Sampling (as grain)” if appropriate considerations of scale-down are being applied.

Finally, if a specific sampling standard were to be developed for tobacco GMO testing, it would be quite prudent to take guidance from the International Standard ISO 7002:1986 “Agricultural food products – Layout for a standard method of sampling from a lot”.

**Practical Approaches**

For all practical purposes in today’s business environment, the determination of transgenic tobacco has the objective to demonstrate its absence from, or under special circumstances its presence in, a given lot of commercial tobacco. This is of overriding influence on the development of sampling plans. Passing judgement based on available test results, the presence of transgenic varieties does not seem to be wide-spread in today’s commercial tobaccos. Transgenic material is only expected in crops from certain areas (in certain varieties and from certain crop years). Consequently, in practice, sampling plans will focus strongly on those tobacco lots which are suspected to contain transgenic material.

On the other side, one should not be too reliant upon available information regarding the presence or absence of transgenic material in commercial tobacco. Therefore, duty of care requires also a screening-type approach to sampling, generally within the limits of resources and practicability.
Regarding sampling procedures for tobacco lots, there are not yet any binding standards or any generally accepted procedures. However, it is possible to draw conclusions, and derive recommendations, from the experience gathered to date in many laboratories. One has to recognize that generally the transgenic tobacco cannot be expected to be evenly distributed in a lot, frequently not even in a sampling unit, such as a carton or sack. Therefore, if sampling units of concern have been identified, based on information about their origin, it is recommended, as a matter of principle, **to take as many incremental samples as possible**. In practice, however, the number of increments will be limited by technical practicability and by the available testing capabilities. An economical way of examining a large number of incremental samples for the absence of transgenic material is pointed out below.

The examination of **green tobacco** offers an opportunity to assess the presence of transgenic material at an early stage in the supply chain. The testing of small green plants, following germination and before they are fully grown and subsequently mixed up with other product, looks like a very attractive option to ascertain whether a crop will be free from transgenic material or not because the work-up and analysis of this kind of green material is technically simpler than working with cured leaf or directly with seed tissue.

Turning next to **raw (cured) tobacco**, samples may have to be taken from packaged tobacco leaf, from tobacco ribs (quite important in practice), from tobacco dust (out of packages, from the factory floor or in the form of milled material) and from cut tobacco (during processing). Both tobacco ribs, tobacco dust and cut tobacco can be assumed to have been subject to some kind of mixing and blending which offers a certain advantage in terms of representativity but, at the same time, has probably “diluted” any transgenic material present. The collection of samples is easy in practice, and scoops, shovels and tweezers are the preferred collection devices.

From packaged (pressed) tobacco, samples are usually taken by means of long cylindrical samplers with a sharp round end which are driven through the packaged tobacco to produce so-called auger samples (Bohrmuster, échantillons). In case of pressed strips (which often come in cartons of up to 400 kg weight) it is advisable to take, at minimum, one incremental auger sample each in the vertical and the two horizontal directions. If more incremental samples are collected from a carton they should be taken in identical numbers in the three directions and in a surface pattern as widely spread-out as possible – the objective being always to make the sampling process as randomized as possible (9 or 12 increments are desirable). Another rather simple sampling approach is to remove the packaging material from the top surface of the carton and to cut, from the pressed strips, one or several rectangular incremental pieces.

If a larger number of cartons needs to be examined, it is advisable to take the auger incremental samples (or the cut-out pieces) from every fifth or tenth carton. Auger samples or cut-out pieces may also be taken from pressed oriental tobacco leafs which generally come in bales of much smaller size (typically around 30 kg).

Dried commercial tobacco intended for cigar wrapper manufacturing comes in the form of layered whole leafs (bundles). Here, the collection of auger samples or the cutting-out of pieces is only recommended if the intact leaf structure does not have to be preserved. Otherwise it much rather makes sense to untie the leafs and select individual ones for testing. In this case one must be aware of the fact that possible heterogeneity within the lot may lead to problems.
The combination of incremental samples (auger samples, cut-out pieces or particulate samples such as dust, ribs or cut tobacco) to produce the basic sample should be done according to established laboratory practices by cutting, mixing and milling. Care should be taken to ensure that no significant amounts of material are lost during these operations and that representativity is maintained during reduction and sub-sampling steps. Such steps should be kept to a minimum. In the end, most analytical laboratories prefer to receive laboratory samples having weights between 0.5 and 1 kg.

Presently, testing for genetic modifications is much less frequently done on the finished product, cigarettes, or on tobacco seeds although this may become more important in the future. For the sampling of cigarettes, the International Standard ISO 8243:1991 provides reliable guidance.

With respect to seeds, available collection and testing resources will determine sampling intensity. Ideally, the number of incremental samples taken should be as high as the number of individual seed production sites in terms of year, location and variety. Typically, sample size is around 100 mg. To economize the testing process, a relatively large number of seed samples may be combined into one laboratory sample if in fact only proof of absence of transgenic material is required (see below).

From both an economical and practical point of view, it is highly desirable that a standardized procedure for seed sampling should be developed and adopted on a global scale. It would enable analytical laboratories to conduct the analyses in a timely fashion, rather than with a high degree of urgency after the tobacco harvest when the grown crop is close to, or already in the process of, being marketed and commercial decisions have to be made rapidly.

A Few Notes of Advice and Caution

Sampling and analysis are linked inextricably. In spite of this, the task of taking and preparing samples is often assigned to people with insufficient training and experience while the analytical methods are being pushed to ever higher levels of accuracy, precision and sensitivity. But even the most sophisticated analytical results are of limited value if the preceding steps of sampling and sample preparation are based on inadequate strategy and sloppy execution. Therefore, it is absolutely necessary to ensure that those taking and preparing samples are properly trained and that sampling protocols are followed faithfully.

It is always worth emphasizing that good practices in handling samples need to be observed particularly in GMO testing because of the delicacy and sensitivity of the analytical methodology. It must be ensured that the test samples are not contaminated or transformed in any way during sampling, transport or storage. The use of suitable and well-sealing containers and the observation of storage conditions appropriate for tobacco samples is mandatory. Full documentation is required for every sample as well as the clear labeling of all sample containers by code numbers, bar codes or attached label with relevant information. Finally, any samples submitted for analytical examinations as involved as those in GMO testing deserve to be accompanied by a proper sampling report.

Because the analytical methodology of testing for transgenic tobacco is capable to demonstrate the presence of very small amounts, great care needs to be taken to avoid the accidental contamination of lots and – even worse – of samples. Such contamination may
occur under two conditions. If a consignment or lot contains damaged sampling units which potentially contain transgenic tobacco, extreme caution has to be taken that the other sampling units are not contaminated. The remedial action which needs to be taken in such cases depends on the specific situation.

The other “opportunity” for contamination is the whole sampling and sample preparation process itself. Any step in the procedure should be done with particular attention to the possibility of cross-contamination. It is good analytical practice to relegate sample processing (including the preparation of the laboratory samples), the work-up of the test sample and the execution of the analysis itself to separate laboratory rooms. Apparatus, devices and labware used in sampling, mixing and milling should not be interchanged and thoroughly cleaned after each use. The sample containers which necessarily have to be moved between the laboratories should equally be subjected to rigorous maintenance procedures (labeling, cleaning, examination for leaks, etc.)

Some procedures during sampling may result in changes in the sample material and render it less amenable for testing. For example, milling may result in localized heating, which can in some cases affect the subsequent DNA extraction. Freezing, thawing, wetting and drying regimens can also damage the DNA to be extracted. It is wise that the procedures for sample preparation are developed in co-operation with the testing laboratory.

If the testing of commercial lots for transgenic tobacco is performed as a screening procedure, exercising due diligence, it is practical and may fully serve the purpose to do the GMO testing on (some of) the same samples that are prepared anyway for chemical, pesticide and sensory analysis.

In countries with closely watched tobacco variety programs or with stringent regulations regarding cultivation of transgenic crops, including tobacco, it may be justified to assume that no transgenic material is present in a consignment of different lots of raw tobacco or tobacco seeds. However, proof is required. Under these circumstances, it is economical to combine a larger number of incremental samples (20 or more) from the different tobacco or seed lots into one basic sample and test it. If the basic sample proves to be negative, all these tobaccos or seeds may be considered to contain no transgenic material, provided that careful consideration has been given to both the dilution due to sample combination and the limit of detection of the method in use. It must not happen that the concentration of transgenic material in the basic sample drops below the limit of detection as a result of excessive sample combination. However, if the first basic sample is positive, additional basic samples from fewer and fewer incremental samples will have to be prepared and tested until the exact source is identified.

Regulatory agencies have now begun to establish threshold levels for inadvertent commingling of transgenic raw material in food and feed (e.g., 1% being the threshold for food crops in Switzerland). A threshold, if exceeded, is the basis for requiring a GMO-related label on the packaging of a commercial product. On the other side, products which are shown to contain less than a fixed percentage of transgenic material may not require a label of this kind. While no such developments are currently under way for tobacco or tobacco products, they can be imagined to occur at some time in the future. If this in fact would happen, the question of the point of enforcement would become one of highest practical importance – with immediate effects on the choice of appropriate sampling procedures.
References

Although most of these references do not deal specifically with tobacco or tobacco products, the following brochures and papers may be quite worthwhile to read:


III. SAMPLE PREPARATION AND DNA ISOLATION METHODS

Introduction

Genomic DNA can be extracted easily from green leaf of tobacco (N. tabacum), whereas the complex matrix of cured leaf can affect the suitability of DNA for GMO analysis. This review describes some methods of sample preparation and DNA isolation applicable to tobacco, for the provision of DNA suitable for PCR reaction.

Outline of Strategies

Plant tissues contain significant quantities of polysaccharide and phenolic compounds, which can inhibit PCR. It is therefore important that DNA extracts should not be contaminated by these compounds. A number of methods have been used to isolate and purify plant for PCR reaction.

Overview of General Methods

Plant DNA isolation consists of two steps; (i) disruption of the sample tissue to release the DNA, and (ii) purification of the DNA from other compounds. Two basic methods for sample preparation are in common use. In one method, DNA is released by alkali treatment of the samples [1], and this DNA can then be used directly for PCR reaction. In the other method, the samples are ground in liquid nitrogen, and the DNA is subsequently purified from this ground material.
**Alkali DNA Extraction**

The merits of this method are its simplicity and that preparation of the DNA template is performed in a single tube. Plant tissue is briefly boiled in sodium hydroxide, neutralised with buffer, and re-boiled. The extract is then used directly for PCR. However, it has not been confirmed whether this method is applicable to cured tobacco leaf.

**Sample Grinding and DNA Purification**

The alternative approach is that the sample is frozen and ground to fine powder in liquid nitrogen. DNA can be extracted and purified from this powder by several possible methods, three of which are described below:

In one commonly-used method [2] the ground plant tissue is heated in a solution of the detergent CTAB, (cetyltrimethylammonium bromide) and then repeatedly extracted with chloroform. The DNA is then precipitated, washed and used for PCR.

A second common method of DNA purification involves phenol/chloroform extraction. This basic method has been modified successfully for isolation of tobacco DNA for PCR. The sample is heated in an extraction buffer before being repeatedly extracted with a phenol/chloroform mixture. DNA is precipitated from the extract, washed, and treated to remove any RNA present in the isolate.

Many kits are commercially available for DNA isolation from various plant materials. One such, the QIAGEN DNEasy Plant Kit has been used successfully in several laboratories. The method comprises incubation of the sample with extraction buffers followed by repeated column purification, using a set of pre-made buffers and columns supplied with the kit. These should ensure the reproducibility of the method and reduce the potential for sample cross-contamination.

**Overview of Tobacco Specific Methods**

As noted above, the Alkali Treatment preparation method has not been validated for application to cured tobacco leaf, whilst methods using liquid nitrogen-ground samples and subsequent purification can be applied to cured leaf. Modified CTAB, phenol/chloroform, and kit-based extraction methods have been developed for isolation of tobacco DNA for the purposes of PCR, and have been confirmed as applicable to cured tobacco leaf.
Summary

Numerous methods have been developed for the extraction of DNA from plant samples. These methods have been refined to enable the isolation of DNA of suitable quality for PCR analysis, and many examples are available both in the literature and as commercial kits. In order to accommodate cured tobacco leaf as the starting material, certain of these methods have been selected and modified, but their applicability should still be verified in individual laboratories.

References


Detailed Methods

Alkali DNA Extraction Method

1. Collect plant tissue into sterile microfuge tubes, containing 40µl of 0.25M NaOH, using the lid of the tube to sever the sample from the plant.
2. Incubate the samples in a boiling water bath for 30 sec and subsequently neutralize by addition of 40µl of 0.25M HCl, and 20µl of 0.5M Tris-HCl, pH8.0; 0.25% (v/v) Nonidet P-40 (Sigma), before boiling for a further 2 min.
3. Use the samples immediately for PCR.
4. Samples can be kept at 4º for several weeks. Incubate stored samples again for 2 min at 100º prior to PCR.

Note:

Quantities of sample materials for extraction by the following three methods are quoted for fresh green leaf samples. Suitable quantities of ground tobacco must be determined empirically.

Modified CTAB Extraction Method

Using 0.1-0.2 g (wet weight) of plant tissue, will yield approximately 50 µg of DNA.

1. Transfer the sample to a microfuge tube (1.5ml).
2. Add 300µl of 2 % CTAB solution (100mM Tris-HCl, pH 8.0, 20mM EDTA, pH 8.0, 1.4M NaCl, 2 % CTAB) and mix by inversion.
3. Incubate the mixture for 30 min at 65º.
4. Add equal volume of Chloroform / iso-amyl-alcohol (24:1, v/v) and shake gently for 5 min.
5. Centrifuge at 12,000 rpm for 15 min and transfer aqueous phase to a new tube.
6. Repeat steps 4 and 5.
7. Add 1-1.5 volume of 1 % CTAB solution (50mM Tris-HCl, pH 8.0, 10mM EDTA, pH 8.0, 1 % CTAB), mix by inversion, incubate at room temperature for 1 hour, and centrifuge at 8,000 rpm for 10 min.
8. Discard the supernatant and redissolve the precipitate by adding 400µl of 1M CsCl.
9. Precipitate and wash DNA by ethanol precipitation.
Modified Phenol/Chloroform Extraction Method

This method uses 1g (wet weight) of plant tissue (young leaves).

1. Prepare extraction buffer by adding 20µl of 2-mercaptoethanol to 1000µl of 2 x stock extract solution (100mM Tris-HCl, pH 8.0; 20mM EDTA; 1 % SDS; 1.5M NaCl), and preheat to (50º). Add 1 ml to sample and mix.
2. Transfer 600µl of the mixture to a 2ml tube and incubate for 20 min at 50º.
3. Add 500µl of phenol/ chloroform (1:1) to the lysate, mix by vigorously shaking and shake for at least 60 min, at 37-45º.
4. Centrifuge for 20 min at 15000 rpm at 25º and transfer 400µl of the supernatant to a new tube.
5. Add 400µl of 1x stock extract solution (50mM Tris-HCl, pH8.0; 10mM EDTA; 0.5 % SDS; 1.5M NaCl) and 500-µl of phenol/ chloroform to the supernatant and shake for 30 min at 37º.
6. Centrifuge for 30 min at 15000 rpm at 25º and transfer 600µl of the supernatant to a new tube.
7. Add 500µl of chloroform and shake for at least 10 min at room temperature.
8. Centrifuge for 30 min at 15000 rpm at 25º and transfer 450µl of the supernatant to a new tube.
10. Centrifuge for 30 min at 15000 rpm at 25º and discard the supernatant carefully.
11. Wash DNA with 70 % ethanol twice and treat with RNase.
QIAGEN DNeasy Plant Mini Kit Method

The maximum amount of sample material using this kit is 0.1 g (wet weight, young tobacco leaves) which should yield 20-25 µg DNA. Up to 1g (wet weight) of plant tissue, can be extracted using the QIAGEN DNeasy Maxi Kit which should yield 120 µg DNA.

All buffers and columns described below are supplied with the Kit.
1. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw.
2. Add 400µl of Buffer AP1 and 4µl of RNase A solution (100 mg/ml), and vortex vigorously.
3. Incubate the mixture for 10 min at 65º. Mix 2-3 times during incubation by inversion.
4. Add 130µl of Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.
5. Apply the lysate to the QIAshredder spin column in a 2 ml collection tube and centrifuge in a micro centrifuge for 2 min at maximum speed.
6. Transfer flow-through fraction from step 5 to a new tube with out disturbing the cell debris pellet.
7. Add 0.5 volume of Buffer AP3 and 1 volume of ethanol (96-100 %) to the cleared lysate and mix by pipetting.
8. Apply 650-µl of the mixture from step 7, including any precipitate, which may have formed, onto DNeasy mini spin column sitting in a 2ml collection tube. Centrifuge for 1 min at >6000 x g and discard flow-through.
10. Place DNeasy column in a new 2 ml collection tube, add 500µl Buffer AW onto the DNeasy column and centrifuge for 1 min at >6000 x g. Discard flow-through and reuse the collection tube in Step 11.
11. Add 500µl Buffer AW to DNeasy column and centrifuge for 2 min at maximum speed to dry the column membrane.
12. Transfer DNeasy column to a 1.5 ml or 2 ml microfuge tube and pipet 100-µl of preheated (65º) Buffer AE directly onto the DNeasy column membrane. Incubate for 5 min at room temperature and then centrifuge for 1 min at 6000x g to elute.
13. Repeat elution (Step 12) as described.
IV. PCR-BASED SCREENING METHODS

Introduction............................................................................................................................ 23
Outline of Detection Strategies.............................................................................................. 23
Strengths of PCR................................................................................................................ 24
Weaknesses and Limitations.............................................................................................. 24
Overview of General PCR Methods ...................................................................................... 24
  General............................................................................................................................... 24
  DNA Quality ...................................................................................................................... 24
  Primer Design .................................................................................................................... 25
  PCR Reaction..................................................................................................................... 26
  Nested PCR........................................................................................................................ 26
  Multiplex PCR ................................................................................................................... 27
  Gene Targets ...................................................................................................................... 27
    Generic Transgenes........................................................................................................ 27
    Specific Transgenes ....................................................................................................... 29
    Recombinant Transgene Assays .................................................................................... 29
Confirmatory tests.............................................................................................................. 30
  Nested PCR.................................................................................................................... 30
  Restriction Analysis ....................................................................................................... 31
  Probe Hybridisation ....................................................................................................... 31
  Sequencing.....................................................................................................................31
Overview of Tobacco Specific Methods................................................................................ 32
DNA Quality and PCR Reaction ....................................................................................... 32
Gene Targets ...................................................................................................................... 33
  Generic Transgenes........................................................................................................ 33
  Specific Transgenes ....................................................................................................... 34
  Recombinant Transgene Assays .................................................................................... 34
Validation of Results.......................................................................................................... 34
Interpretation...................................................................................................................... 35
Confirmation...................................................................................................................... 35
  Nested PCR.................................................................................................................... 36
  Restriction Analysis ....................................................................................................... 36
  Probe Hybridisation ....................................................................................................... 36
  Sequencing.....................................................................................................................36
Summary................................................................................................................................ 36
References.............................................................................................................................. 40
Primer Sequences................................................................................................................... 40
Introduction

Genetic modification of tobacco has been an established laboratory procedure for many years. The process of producing GM plants, through the direct introduction of transgenes, is largely independent of the genes involved, and there are common features in the structures of the gene constructs employed. Hence, despite there being a variety of transgenes in use, generic strategies can be devised for the detection of most genetic modifications.

Detection of transgenes, which may not be being expressed at that time, can only be achieved by analysis of plant DNA. By their very nature, transgenes are novel, and can be distinguished from the surrounding tobacco genome, but at the practical level this requires either some knowledge of the inserted DNA, or a guess at the likely candidate sequences.

Transgenes conferring resistance to viral and bacterial diseases of tobacco are known to have been employed and are therefore possible candidates for screening. Two or more transgenes may be introduced simultaneously, and one of these may be a "selectable marker", necessary only during the laboratory stage, but usually still present in the final plant, such as the nptII gene. In addition, other DNA features found in most modifications include the 35S promoter from Cauliflower Mosaic Virus, and the nos promoter and nos terminator from Agrobacterium tumefaciens.

Methods for detection of transgenes in tobacco have been developed in a number of laboratories and this review summarises the methods used. Where possible, practical details have been attributed to their originators. In other cases where information is proprietary or apocryphal, a general description has been given.

Outline of Detection Strategies

The most common methodology employed for GM screening is PCR-based detection of transgenes followed by gel electrophoresis and comparison with standard samples. The process uses the enzyme Taq DNA Polymerase to amplify minute quantities of transgene DNA from plant material to a detectable level.

Most methods for transgene detection in plant products have been devised for the analysis of food materials [1]. In general, two strategies are adopted, based either on the detection of one or more "generic" transgene sequence (usually the 35S promoter), or on screening for a range of specific sequences.

As a complication, the transgenes being sought may comprise segments of DNA derived from viruses or bacteria that occur in the natural environment. Detection of these sequences in a sample may then be misinterpreted as indicating GM plants. Even RNA viruses (e.g. TMV, CMV), may pose a problem, since under certain circumstances Taq DNA Polymerase is capable of converting RNA to DNA [2,3], which could be detected in the subsequent assay. However, this potential ambiguity can be avoided by judicious selection of the target regions of transgenes being detected (See "Recombinant Transgenes" below), and a reliable diagnosis can be made.
Strengths of PCR

A major advantage of a PCR-based detection-strategy is that it is extremely sensitive. It can be highly specific, whilst the basic test process is similar whichever genetic modification is being screened for. PCR can detect transgenes whether or not they are being expressed and it can even detect partial gene sequences and non-coding regions.

Weaknesses and Limitations

The sensitivity of PCR means that the process is sensitive to contamination, and its high specificity can also lead to a failure to detect slight variants of the target sequences. Quantitation of GM material present in samples requires detailed procedures and will be addressed elsewhere [4]. A threshold of detection must be determined, and zero tolerance is scientifically unverifiable at present.

Overview of General PCR Methods

General

PCR is a common procedure in most analytical laboratories and basic operational procedures are well established, and will not be described here. These procedures are designed to eliminate the possibility of cross-contamination between test samples and controls. In general PCR screening should be conducted in a separate location to sample preparation, and any other work with transgenes. Some laboratories decontaminate working surfaces and apparatus between runs using ultraviolet light [5], or chemical reagents such as DNA Zap [6].

DNA Quality

As described in Section III, a critical aspect of the testing procedure is the isolation of DNA of suitable quality for PCR. The process of harvesting, storing, and processing plant materials introduces many abuses to the DNA which ideally the molecular biologist would avoid, including exposure to intracellular enzymes and chemicals during natural senescence, heating, freezing and thawing, wetting and drying. These lead to DNA fragmentation, and so all DNA preparations should be assessed for their fragment size profile, usually by visual appearance on gel electrophoresis. One advantage of PCR, however, is that it can utilise relatively short DNA fragments.

The PCR process is also prone to inhibition by other materials co-extracted from the matrix, including metals, carbohydrates, phenolics, and salts. This is particularly a problem with food analysis. Most DNA extraction methods address these problems, often using silica-based purification stages, whilst use of strong denaturants in the process may also be advantageous.
DNA quality is normally assessed by a PCR test for an endogenous DNA sequence, which is present in the plant genome whether GM or not. This screen verifies the "PCR quality" status of the extracted DNA and guards against false negatives. The endogenous target should be a longer sequence than the transgene targets to ensure that genomic DNA fragments of a sufficient length are present. In some laboratories the PCR quality test is conducted simultaneously with the transgene test using multiplex PCR (Described below). Most laboratories test for the PCR of a chloroplast sequence, although some labs use a nuclear DNA sequence. This sequence could be specific for a particular crop plant, such as a Brassica seed protein gene or a soybean lectin gene, or alternatively a general "plant" sequence.

The chloroplast sequence test is well established [1,5,7,8] though a nuclear DNA assay has some advantages in that it specifically evaluates DNA from the cellular location where putative transgenes would be located. Nuclear and chloroplast DNA may differ in their ease of extraction, and may degrade to different extents during curing. Also, unlike chloroplast sequences, the nuclear sequences are present at a low copy number per genome, similar to that expected for transgenes, and the assay is therefore conducted at the appropriate level of sensitivity to detect any transgenes. The respective strengths and weaknesses of these two tests are illustrated in Table 1.

**Table 1.** Comparative advantages and disadvantages of two types of PCR ability tests using different endogenous genes.

<table>
<thead>
<tr>
<th>Test</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chloroplast Gene</strong></td>
<td>Established Process</td>
<td>Not representative of integrity of (nucleus-located) transgenes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Different sensitivity to transgene detection</td>
</tr>
<tr>
<td><strong>Nuclear Gene</strong></td>
<td>Representative of integrity of (nucleus-located) transgenes</td>
<td>Not standardized</td>
</tr>
<tr>
<td></td>
<td>Similar sensitivity to transgene detection</td>
<td></td>
</tr>
</tbody>
</table>

**Primer Design**

Primer design is a crucial aspect of a successful PCR, but this has not always been the first consideration in the analysis of processed plant material, and primers have often been selected empirically. If the luxury of sufficient sequence information is available, some key elements in primer design are:

- Maximise the primer specificity
- Use primers of 18 - 30 nucleotides in length
- Avoid GC rich, repetitive, or self-complementary sequences
- Use a stable 5’ end and a less stable, very specific 3’ end to primer
- Select a high annealing temperature if possible
- Avoid primer pairs capable of binding to each other (primer-dimers)
- Keep the expected PCR fragment as short as possible
PCR Reaction

The basic PCR process consists of multiple components and reaction steps. Independent development in different labs has resulted in widely varying parameters, some of which are outlined below.

PCR is usually conducted in microtubes or microtitre plates, and reaction volumes vary from 10 to 100µl. The quantity of template DNA used also varies considerably. PCR reaction schemes differ with respect to times, temperatures, and numbers of amplification cycles, often for the same assay in different laboratories. For some assays conditions can be standardised which enables multiple assays to be conducted simultaneously on the same thermocycler, and also permits multiplex PCR. This standardisation however may mean compromise which can lead to sub-optimal reaction in some cases.

Most PCR tests are assessed by agarose gel electrophoresis, and results are scored visually as the presence or absence of a DNA fragment of the appropriate size. Certain PCR assays which yield a single, well-characterised product can be scored without the need for resolution by electrophoresis.

Nested PCR

A second round of PCR may be performed to add specificity to the assay (see Confirmation), or to increase the sensitivity. This "nested PCR" utilises a second pair of primers directed to the same target sequence, but internal to the first pair. These may or may not overlap the first pair of primers.

Nested PCR can be conducted by
(i) sampling a small aliquot from the first PCR into a second reaction mix,
(ii) adding new primers part way through the cycle, or
(iii) decreasing the annealing temperature part way through the cycle to enable the second pair of primers to come into play. This method precludes the need to re-open the reaction tube (potentially allowing cross-contamination), but does require careful primer design.

Table 2. Advantages and disadvantages of Nested PCR compared to basic PCR

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased sensitivity</td>
<td>Increased opportunity for contamination</td>
</tr>
<tr>
<td>Increased specificity</td>
<td>Increase may not be significant</td>
</tr>
<tr>
<td></td>
<td>More complex and lengthy</td>
</tr>
</tbody>
</table>
**Multiplex PCR**

Multiplex PCR entails the simultaneous PCR of two or more different target sequences in the same reaction tube. This can be used to save time and resources when screening for several transgene sequences, and also to verify the effectiveness of the PCR reaction by combining the "PCR-quality" and transgene screens in a single reaction.

A drawback of multiplex PCR is the potential competition for reagents between the two reactions, especially if the two targets are present in significantly different concentrations. Hence a GM target at low concentration may be "starved" of reagents and fail to amplify to a detectable level. This may be exacerbated by differing efficiencies of the two sets of primers. The optimal annealing temperature for each primer pair may differ and a multiplex reaction is of necessity conducted under compromise conditions. The two reactions may not be equally effective under such conditions.

When the copy numbers of the two targets differ significantly, as is the case when using chloroplast and nuclear sequences, empirically adjusting the ratio of primer concentrations can be used to compensate, but primer ratios established in this way may not be universally useful for different sample materials (leaf, stem, different tobacco varieties, and different degrees of degradation).

Comparisons of PCR efficiency are dependent upon the PCR products being of similar size. However, multiplex PCR demands that products differ in order to be resolved from each other on electrophoresis gels following reaction. Multiplex PCR also complicates any non-gel-based PCR detection method, since post-reaction separation of the products is required.

**Table 3.** Advantages and disadvantages of Multiplex PCR compared to basic PCR

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Offers internal PCR control</td>
<td>Templates may compete</td>
</tr>
<tr>
<td>Saves resources</td>
<td>PCR may be non-optimised</td>
</tr>
<tr>
<td></td>
<td>Requires gel analysis</td>
</tr>
</tbody>
</table>

**Gene Targets**

**Generic Transgenes**

The ideal PCR target is a generic sequence, found in all GM plants but not in the native plant nor in associated microbes. Ideally it should not occur in any processing additives either. Current generic targets include the 35S-promoter (most used), the nos-promoter, the nos-terminator, and the nptII gene. In addition, transfer DNA (T-DNA) sequences from A. tumefaciens-derived transformation vectors may be useful for transgenic plant screening. However, a survey of such "generic" targets in a selection of existing transgenic food materials indicates that even using combinations of targets does not guarantee infallible detection of genetic modification [See Table 4.].
Analyses will soon need to include second generation targets, including novel promoters for controlled expression. It is also likely that in future, vector sequences and especially antibiotic markers will be removed from the final product.

**Table 4.** Presence of "generic" transgenes in 28 different transgenic plants currently approved or seeking approval for release in Europe. [Data summarized from Reference 8.]

<table>
<thead>
<tr>
<th>Transgenic sequence</th>
<th>Plants containing one or more</th>
<th>GM plants undetectable using this screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S</td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td>nos-terminator</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>35S, and nos-terminator</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>NPTII</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>35S, and NPTII</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>35S, nos-terminator, and NPTII</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>nos-promoter</td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>35S, and nos-promoter</td>
<td>25</td>
<td>3</td>
</tr>
</tbody>
</table>

35S Promoter

This test is used in most laboratories screening GM plants and relies on the 35S promoter being the most commonly used sequence in GM plants. There are natural variants of this sequence and also variations in the detailed assembly of transgene constructs, so it must be confirmed that any primers used are both effective and equally efficient in all cases.

*Neomycin phosphotransferase II (nptII, Kanamycin resistance)*

The *nptII* gene is found in most early transformation vectors, and is often used in GM verification during the process of producing transgenic plants. However, since the gene is of bacterial origin, it is may be unreliable because of potential bacterial contamination [5].

*Hygromycin phosphotransferase*

The hygromycin phosphotransferase gene is used in some transformation vectors as an alternative selectable marker to *nptII*. A screen for this marker has been developed for food analysis [7].
nos-promoter and nos-terminator (*nopaline synthase* promoter and terminator)

These sequences are also common features of many transformation vectors. They are derived from *Agrobacterium tumefaciens*, a soil bacterium, and therefore may not be ideal for assays on roots [8] or other soil-carrying material. However, strains of *Agrobacterium tumefaciens* found naturally in soil are often avirulent, i.e. they lack the Ti-plasmid that carries the T-DNA and its oncogenes (e.g. *nos*).

**Specific Transgenes**

In addition to the screening for generic sequences, a number of laboratories have developed tests for specific transgenes, based upon well-characterised gene modifications. Such target genes include:

- cry1A gene (Bt - insect resistance)
- EPSPS gene (Glyphosate/RoundUp resistance)
- bar gene (BASTA resistance)
- polygalacturonase gene (delayed fruit ripening)

**Recombinant Transgene Assays**

When the transgenes under scrutiny are derived from micro-organisms present in the environment, false positives are a potential hazard. However, transgenes comprise microbe-derived sequences "spliced" to other DNA elements (promoters and terminators) in combinations which do not occur in the native micro-organism. Hence specific DNA junction regions occur, and by detecting these junctions, a reliable diagnosis can be made. Presence of the junction sequence confirms GM status of the plant for the particular transgene construct, since neither homologous sequences from the unmodified plant genome, nor genes from contaminating bacteria or virus will give a positive signals in these tests.
Table 5. Advantages and disadvantages of different transgene targets.

<table>
<thead>
<tr>
<th>Transgene Target</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S Promoter</td>
<td>Most common transgene</td>
<td>Not 100% generic</td>
</tr>
<tr>
<td></td>
<td>Established process</td>
<td>May become obsolete soon</td>
</tr>
<tr>
<td></td>
<td>Viral source limited to certain crops</td>
<td>Some endogenous homologies</td>
</tr>
<tr>
<td>nptII</td>
<td>Common marker transgene</td>
<td>Not 100% generic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>May become obsolete soon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occurs in associated bacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some endogenous homologies</td>
</tr>
<tr>
<td>nos Terminator</td>
<td>Common transgene</td>
<td>Not 100% generic</td>
</tr>
<tr>
<td></td>
<td>Established process</td>
<td>Occurs in associated bacteria</td>
</tr>
<tr>
<td>Specific transgenes</td>
<td>Specific</td>
<td>Limited use</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Can occur in &quot;environment&quot;</td>
</tr>
<tr>
<td>Recombinant transgenes</td>
<td>Highly specific</td>
<td>Very limited use</td>
</tr>
<tr>
<td></td>
<td>Cannot occur in &quot;environment&quot;</td>
<td>Requires information</td>
</tr>
</tbody>
</table>

**Confirmatory tests**

Test samples which have been found positive for transgene sequences by PCR assay can be further analysed to provide information on the exact nature of the genetic modification. Several approaches are possible:

**Nested PCR**

Any of the PCR assays used in the basic analysis can be enhanced by further rounds of PCR to improve the specificity of detection. The method can employ either one (semi-nested PCR) or two (nested PCR) additional primers which will identify further regions of the transgene sequence within the PCR fragments already produced. A positive result is indicated by the production of a new PCR fragment of a defined length. Depending on the nature of the nested reaction, either two or one (the smaller) PCR products may be detected following the reaction. The method thereby confirms the presence and identity of the transgene by showing that the DNA fragment detected in the initial assay possesses the expected transgene sequence at various internal regions.
Restriction Analysis

This approach also bases the confirmation of transgene identity upon the detection of specific internal sequences. In many cases the full DNA sequence of the candidate transgene is known, which will include the identification of the presence and position of restriction endonuclease sites within it. By challenging the PCR fragment obtained in the initial transgene screen with one or more restriction endonucleases, the fragment is cleaved into smaller pieces of defined length. The presence of these cleavage sites within the fragment can then be used to confirm the identity of the transgene sequence. When using this confirmation assay, it is important that the initial PCR product is purified to remove reagents which may inhibit the action of the restriction endonucleases and so lead to false negative results [5].

Probe Hybridisation

Cloned copies of the transgenes or synthetic oligonucleotides can be used to confirm the identity of PCR fragments by Southern hybridisation. This method has advantages over the Nested PCR and Restriction Analysis approaches in that it confirms identity (or at least homology) along the full length of the transgene sequence rather than at short isolated sections. The hybridisation procedure can also be adjusted to tolerate certain variations in the transgene sequence and so can accommodate variants, without requiring foreknowledge of the variant sequence. It is important that the appropriate controls are included in this assay to ensure that the hybridisation is conducted under appropriate levels of stringency.

Sequencing

Whilst the previous methods depend upon the detected fragment possessing a known sequence, DNA sequencing could identify the sequence of the target analyte de novo, based solely upon homology at one or both ends of the fragment, and can therefore resolve doubts on uncertain results. Primer sequences used for the PCR assay may also be employed for sequencing of either the resultant PCR product, or even the genomic DNA itself. Since the transgene target sequences are relatively short, sequencing is not a problem if suitable quality template DNA is available.

Sequencing provides entire and absolute information on the transgene at the nucleotide level, and therefore can determine the precise variant of the transgene employed, including any artefacts introduced during vector construction, etc. The full identity and therefore possibly even the (laboratory) origin of the transgene can be determined. Sequencing can also provide information on the site of insertion of the transgene into the plant genome, and whether there are several insertions in the same plant. This information can act as a "fingerprint" for the transgenic plant and can discriminate between plants which carry the same transgenes, but which are derived from individual transformation events. Again this knowledge can lead to the identification of "pedigrees" and histories for transgenic plant material.
Table 6. Advantages and Disadvantages of different Confirmatory tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested PCR</td>
<td>Increased specificity</td>
<td>Increase may not be significant</td>
</tr>
<tr>
<td></td>
<td>Established Process</td>
<td>Not independent of first test</td>
</tr>
<tr>
<td>Restriction Analysis</td>
<td>Increased specificity</td>
<td>Increase may not be significant</td>
</tr>
<tr>
<td></td>
<td>Established Process</td>
<td>Requires information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prone to inhibition</td>
</tr>
<tr>
<td>Probe Hybridisation</td>
<td>Assesses whole of transgene</td>
<td>Homology, not identity</td>
</tr>
<tr>
<td></td>
<td>Tolerant of minor variation</td>
<td>More complex technology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires DNA probe</td>
</tr>
<tr>
<td>Sequencing</td>
<td>Absolute identity</td>
<td>More complex technology</td>
</tr>
<tr>
<td></td>
<td>Can determine variants</td>
<td>Not routinely employed</td>
</tr>
<tr>
<td></td>
<td>Can distinguish transformation events</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Requires no additional info</td>
<td></td>
</tr>
</tbody>
</table>

Overview of Tobacco Specific Methods

General PCR methods for detection of GM material have been developed in by several laboratories for application to cured tobacco, and although most protocols remain proprietary, many are largely similar to the method of Pietsch et al [1].

DNA Quality and PCR Reaction

The process of curing tobacco leaf causes much degradation of the genomic DNA and produces many chemical compounds which may inhibit the PCR reaction. Removal of these compounds during the DNA preparation stage is important for successful PCR.

The quantity of template DNA (tobacco) used in the PCR ranges from 5 - 100ng. Apocryphal data suggests that more template DNA yields more numerous positive results, though this may not be comparable between laboratories.

Primer concentrations used vary from 2 - 10.0 µM. In practice, primer lengths are normally around 20 - 25 nucleotides (the shortest reported being 16 [1]), and generally primers pairs are of approximately equal length (± 2 nucleotides). More variable is the GC content of the primers, which although commonly 50-55%, can range from 45 to 65% (with the lowest being 36%). In many instances pairs of primers are significantly mismatched with regard to their GC content.
PCR reaction schemes are broadly similar, reaction times varying with the thermocycler used, and ramping rates being set as fast as possible. Primer annealing temperatures for most assays are standardised at approximately 55°, irrespective of the primer characteristics. This enables multiple assays to be conducted simultaneously, and permits multiplex PCR. In some labs, individual PCR tests are optimised with respect to annealing temperature.

Most assays use 30-35 amplification cycles, although some labs use particular assays of 45-50 cycles. This may increase the sensitivity of the test, but care is necessary in these extended runs as the effect of minor contamination or PCR artefacts is significantly amplified.

PCR results are assessed by gel electrophoresis (1.4 - 4.0% agarose). Ideally a negative result is indicated by total absence of PCR product, though some tests require only the absence of a product of a particular diagnostic size. In some labs an arbitrary threshold is set before a result is called positive (e.g. greater than 2% of the intensity of the positive control).

**Gene Targets**

**Generic Transgenes**

**35S Promoter**

This test is used in all laboratories screening tobacco. Homologous sequences have also been detected in non-transgenic tobacco (or possibly from accompanying bacterial, fungal, and viral genomes present on the harvested leaf).

Most laboratories use the primers described by Pietsch et al [1] (see Appendix), which give rise to a 195bp PCR product. Other primers are used in some screening laboratories, often resulting in larger PCR products (300-400bp).

**Neomycin phosphotransferase II (nptII, Kanamycin resistance)**

This test is used in some laboratories, but it may be unreliable for cured leaf due to bacterial contamination [5]. Presence of nptII-homologous sequences have also been found in the unmodified tobacco genome. The test is used however for screening green leaf and seedlings [5].

Several different sets of primers have been used in NPTII screening, giving rise to products ranging in size from 400 - 800 bp. Those used by Pietsch et al [1] produce a much smaller PCR product of 173bp.

The *hygromycin phosphotransferase* gene is not commonly used in screening tobacco samples as yet.
nos-promoter and nos-terminator (nopaline synthase promoter and terminator)

These sequences are commonly used in screening tobacco. Since they are derived from a soil bacterium they may not be ideal, but although tobacco can indeed be a host to A. tumefaciens, the bacterium is rarely found on tobacco crops. This suggests that the likelihood of finding A. tumefaciens nos sequences on non-GM tobacco leaves is low.

Specific Transgenes

Tests have been developed for several specific transgenes, based upon intelligence that such modifications have been used in tobacco. The following genes have been targeted:

- TMV coat protein gene
- CMV coat protein gene
- PVY coat protein gene
- TMV 54kD gene
- Bt gene (cry1A)

Recombinant Transgene Assays

The risk of non-cruciferous plants being infected with CaMV is extremely low, although related viruses do infect tobacco. There is however a problem when screening for transgene sequences derived from soil bacteria, and also natural pathogens such as TMV and CMV. Even if a native virus does not contain DNA, the viral RNA genome may be converted to a form detectable by the PCR (as described above).

Assays have been developed to detect promoter-coding region junctions, including 35S-TMV and nos-nptII, which are the most common constructs using these genes, and 35S-chloroplast transit sequence [9]. Presence of the junction sequence confirms GM status of the tobacco for that particular transgene construct.

Validation of Results

A positive PCR result only means that a product has been successfully amplified, but the tobacco sample template may not necessarily be the source. Likewise, a negative PCR result only indicates that a product has not been amplified. It does not necessarily imply that the transgene is not present. These problems are addressed by the use of duplicate samples and appropriate controls. Each PCR run performed includes the following controls:

- Verified positive control.
- Verified negative cured leaf sample extract.
- No-DNA blank controls
In all assays, it is important that the best available controls are employed to validate the result. Ideally the positive control comprises a sample of DNA isolated from GM tobacco leaf containing the transgene(s) being assayed, and which has been cured, treated, and extracted in a comparable manner to the test samples. However, verified cured GM tobacco is not always available for some of the transgenes being screened, and the exact molecular identity of the potential GM sample may not be known. If the GM material is available in another form (e.g., seed), this can be used to produce a positive control DNA sample prepared from field grown air-dried leaf or even fresh seedling material. As a final option, DNA from the original transformation vector used to produce the GM plants may be used. However, these latter options are much less desirable as they are hardly comparable to the test samples.

Negative controls comprise non-transgenic cured tobacco DNA, and some laboratories also include "extraction blanks", i.e. dummy samples passed through the extraction process, but containing no starting sample material.

All control samples are prepared and evaluated by the same procedures as used for test samples. In the PCR tests, blank buffer mixes (No DNA) are also included.

Results from the PCR tests are only accepted if all control samples perform as expected. Results are usually rejected and the analyses repeated if PCR fragments are weak, or if several bands are seen. However, Pietsch et al [1] found several spurious PCR products from 35S and nptII assays of plant material, and these were ignored. Negative results ideally require the total absence of PCR product, although in some cases a very faint product is observed but discounted. This is presumably indicative of a low level of contamination in the laboratory.

**Interpretation**

Interpretation of the analysis can depend upon which PCR tests are applied. When only a single test (e.g. 35S) is performed, the result depends solely on the outcome of this test. If several sequences are screened, it is possible that conflicting results may be observed, yet there is no common course of action when this situation arises. Some laboratories retest the samples, some reject the samples if any test is positive, and others form a consensus based on the distribution of positive and negative results. The congruency of results depends on the exact construct present, and may be affected by there having been some re-arrangement of the transgenes in vivo. Results must be coherent and internally consistent, and compatible with any intelligence on the sampling regime.

**Confirmation**

Test samples which have tested positive are usually subjected to further analysis. In practice only Nested PCR and Restriction Analysis are used routinely.
Nested PCR

This is conducted as described above. The process adds little to the original specificity, and in practice when a PCR product is detected, its identity is usually "confirmed" by nested PCR. A more meaningful question to be resolved may be the source of the transgene, i.e. did it originate in the sample or has contamination occurred.

Restriction Analysis

This approach has been used to verify the 35S and nos-terminator assays [8]. Digestion of the 35S fragment with the restriction enzyme Asp700 (XmnI), and the nos-terminator fragment with NsiI yields diagnostic bands, which can be resolved on agarose gels. Other restriction sites are present in these fragments and have also been used in verification assays.

Probe Hybridisation

Confirmation by hybridisation is offered by some testing laboratories, and is recommended in food analysis [7,9]. A commercial kit is available for the detection of PVY infection using PCR and Oligonucleotide Hybridisation [10], and this might be applicable as a transgene screen.

Sequencing

Transgene sequencing is not being conducted routinely by any laboratory analysing tobacco. An unresolved problem with all confirmation assays is the action to be taken following an unconfirmed result. There is no common response to this situation. It must be borne in mind that a single nucleotide change due to a PCR-introduced mutation, or to a variant form of the transgene sequence would prevent reaction in both Nested PCR and Restriction Analysis.

Summary

Effective methods for PCR-based screening of transgenes in tobacco have been developed, using where appropriate similar technology introduced for food analysis. There is a common basic methodology in most laboratories, although details of the process are not standardised. However, differing strategies have been adopted as regards the choice of transgene targets, the criteria of validation, and the interpretation of results. This is in part due to the limited shared intelligence on potential targets and the dearth of control samples, but also to the fact...
that the consequential action following a screening run may dictate precisely what technical information is required. A number of tobacco-specific issues remain, in addition to the technical considerations, not least of these being the definition of tolerance limits for the levels of GM material in tobacco.
Table 7. A summary of the features of a PCR-based screening process, identifying the merits and limitations of each step.

<table>
<thead>
<tr>
<th>Basic PCR Process</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA relatively robust to curing process</td>
<td>DNA quality can vary with source</td>
<td></td>
</tr>
<tr>
<td>Degraded DNA can be used</td>
<td>Matrix affects the process</td>
<td></td>
</tr>
<tr>
<td>High sensitivity</td>
<td>Sensitive to contamination</td>
<td></td>
</tr>
<tr>
<td>Single basic process for all tests</td>
<td>Can detect &quot;environmental&quot; DNA</td>
<td></td>
</tr>
<tr>
<td>Can be automated</td>
<td>Not quantitative (Basic process)</td>
<td></td>
</tr>
<tr>
<td>Permits physical isolation of transgene</td>
<td>Sensitive to parameter changes - cycle no, concentrations</td>
<td></td>
</tr>
<tr>
<td>Independent of expression</td>
<td>May miss minor variants</td>
<td></td>
</tr>
<tr>
<td>Can use non-coding sequences</td>
<td>Detection threshold must be defined</td>
<td></td>
</tr>
<tr>
<td>Can use partial genes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR Refinements</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested PCR</td>
<td>Increased sensitivity</td>
<td>Increased opportunity for contamination</td>
</tr>
<tr>
<td></td>
<td>Increased specificity</td>
<td>May not be significant increase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>More complex/lengthy</td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td>Internal PCR control</td>
<td>Templates may compete</td>
</tr>
<tr>
<td></td>
<td>Saves resources</td>
<td>PCR may be non-optimised</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires gel analysis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR Ability Test</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast gene</td>
<td>Established process</td>
<td>Not representative of integrity of (nucleus-located) transgenes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Different sensitivity to transgene detection</td>
</tr>
<tr>
<td>Nuclear gene</td>
<td>Representative of integrity of (nucleus-located) transgenes</td>
<td>Not standardised</td>
</tr>
<tr>
<td></td>
<td>Similar sensitivity to transgene detection</td>
<td></td>
</tr>
<tr>
<td>Transgene Screens</td>
<td>Advantages</td>
<td>Disadvantages</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>35S Promoter</strong></td>
<td>Most common transgene</td>
<td>Not 100% generic</td>
</tr>
<tr>
<td></td>
<td>Established process</td>
<td>May become obsolete soon</td>
</tr>
<tr>
<td></td>
<td>No &quot;environmental&quot; source (when screening tobacco)</td>
<td>Some endogenous homologies</td>
</tr>
<tr>
<td><strong>nptII</strong></td>
<td>Common marker transgene</td>
<td>Not 100% generic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>May become obsolete soon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occurs in associated bacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some endogenous homologies</td>
</tr>
<tr>
<td><strong>nos Terminator</strong></td>
<td>Common transgene</td>
<td>Not 100% generic</td>
</tr>
<tr>
<td></td>
<td>Established process</td>
<td>Occurs in associated bacteria</td>
</tr>
<tr>
<td><strong>Specific transgenes</strong></td>
<td>Specific</td>
<td>Limited use</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Can occur in &quot;environment&quot;</td>
</tr>
<tr>
<td><strong>Recombinant transgenes</strong></td>
<td>Highly specific</td>
<td>Very limited use</td>
</tr>
<tr>
<td></td>
<td>Cannot occur in &quot;environment&quot;</td>
<td>Requires information</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Confirmatory Tests</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nested PCR</strong></td>
<td>Increased specificity</td>
<td>May not be significant increase</td>
</tr>
<tr>
<td></td>
<td>Established process</td>
<td>Not independent of first test</td>
</tr>
<tr>
<td><strong>Restriction Analysis</strong></td>
<td>Increased specificity</td>
<td>May not be significant increase</td>
</tr>
<tr>
<td></td>
<td>Established process</td>
<td>Requires information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prone to inhibition</td>
</tr>
<tr>
<td><strong>Probe Hybridisation</strong></td>
<td>Assesses whole of transgene</td>
<td>Homology not identity</td>
</tr>
<tr>
<td></td>
<td>Tolerant of minor variation</td>
<td>More complex technology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires DNA probe</td>
</tr>
<tr>
<td><strong>Sequencing</strong></td>
<td>Absolute identity</td>
<td>More complex technology</td>
</tr>
<tr>
<td></td>
<td>Can determine variants</td>
<td>Not routinely employed</td>
</tr>
<tr>
<td></td>
<td>Can distinguish transformation events</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Requires no additional info</td>
<td></td>
</tr>
</tbody>
</table>
References


4. This report, Section V, Semi-quantitative and quantitative PCR methods.

5. This report, Appendix III, Chinese Method for Screening GM Tobacco.

6. Ambion Inc., Austin, TX, USA


10. PE Applied Biosystems, Foster City, CA, USA: http://www.pebio.com/

**Primer Sequences**

**Chloroplast** [1,7,8]

5’ CGA AAT CGG TAG ACG CTA CG  
5’ GGG GAT AGA GGG ACT TGA AC  

Yield a 550bp product, i.e. longer than most transgene targets.

**35S** [7,8,11]

5’ GCT CCT ACA AAT GCC ATC A  
5’ GAT AGT GGG ATT GTG CGT CA  

Yield a 195bp product.

**nptII** [1]

5’ GGA TCT CCT GTC ATC T  
5’ GAT CAT CCT GAT CGA C  

Yield a 173bp product

**nos-terminator** [1,8,11]

5’ GAA TCC TGT TGC CGG TCT TG  
5’ TTA TCC TAG TTT GCG CGC TA  

Yield a 180bp product.

**Hygromycin phosphotransferase** [7]

5’ CGC CGA TGG TTT CTA CAA  
5’ GGC GTC GGT TTC CAC TAT  

Yield a 839 bp product
Introduction

Modern biotechnology is rapidly entering the production of agricultural products, and consequently food, provoking an increasing consumer awareness, particularly in the European Union. Regulatory oversight on the release of genetically modified organisms (GMOs) into the environment and mandatory labelling of products containing GMOs has been established in the EU and other countries. Labelling obligations will depend on the amount of GMO contained in the product. It is thus necessary to quantify exactly the amount of GMO present in a product, through the analysis of deoxyribonucleic acid (DNA) or proteins.

Since the discovery of DNA in 1953 [1], different methods for the detection of this molecule have been developed. One method is the polymerase chain reaction (PCR), which was described for the first time in 1985. In 1993 Kary B. Mullis received the Nobel Prize in chemistry for the invention of this technique [2]. Because of its extraordinarily high sensitivity, PCR is now one of the most popular DNA detection methods, used in many different applications, including the detection of GM-materials.
However, PCR in this simple format is a qualitative detection method, giving only poor quantitative information. Due to the exponential nature of PCR, small variations in a number of experimental variables can greatly influence the final result [3,4,5]. Since the early 90’s, PCR based techniques have been developed to overcome some of the problems associated with quantification of nucleic acids by PCR.

This section describes the status of technical progress of quantitative PCR and its applications to GMO analysis.

**Outline of Strategies**

**Background**

To understand the problems associated with quantitative PCR, a mathematical evaluation of the reaction must be made. In order to describe PCR with a mathematical equation the principle of the reaction should be clear; the PCR is a succession of cycles where, in each cycle, the amount of target molecules is doubled. Mathematically this can be expressed as:

\[
N_n = N_0 \cdot 2^n
\]

where:

- \(N_n\) = number of amplified target molecules in cycle \(n\)
- \(N_0\) = initial number of target molecules
- \(n\) = number of cycles

However, this equation is not a realistic presentation of the reality, as only a proportion of the target molecules will be replicated in each cycle. In order to correct for this phenomenon a parameter, which represents the efficiency (\(E\)) of the replication, is introduced into the equation.

\[
N_n = N_0 \cdot (1 + E)^n
\]

where:

- \(E\) = the efficiency of the reaction representing the fraction of target molecules which are replicated in a cycle

Unfortunately, the efficiency is not a constant parameter but varies between different reactions and even within one reaction. These variations have different impacts on the possibility to use the PCR as a quantitative tool. The variation of efficiency within one reaction is often observed in the later cycles of the PCR. In general the efficiency decreases with increasing cycle number until the amplification reaches the maximum product yield known as the plateau phase [5,6].

Several reasons can be given for the decrease in efficiency:

- Due to the high product concentration, primers compete less effectively with template reannealing [7]
- Denaturation efficiency is reduced in later cycles [5,6]
- Primer concentration decreases [6]
- The ratio of enzyme (DNA polymerase) to product (target with primer) decreases exponentially with the exponential increase of the product [7]
- The DNA polymerase becomes thermally inactivated during later cycles [5,6]
- One or more of the components necessary for the reaction become limiting [5]
- Pyrophosphates (inhibitors of DNA polymerase activity) accumulate [5]
To clarify the impact of a decreasing efficiency, a mathematical approximation of this phenomenon can be introduced into the mathematical model for the PCR. The following equation can be used to describe the relation between the efficiency and a given cycle number:

\[ E_n = E_1 - \alpha \cdot \frac{N_n}{N_{\text{max}}} \quad (3) \]

where:
- \( E_n \) = efficiency at cycle \( n \)
- \( E_1 \) = efficiency at the first cycle
- \( \alpha \) = positive parameter > \( E_1 \) which might be a function of several other parameters

It should be clear that this equation is an approximation and may not quantitatively reflect the decrease of \( E \) with the number of cycles, a phenomenon that depends on many factors. It is, however, sufficiently close to reality to clarify the plateau phenomenon [6]. By combining Equations 2 and 3, a more correct equation for product formation by PCR can be found:

\[ N_n = N_0 \cdot (1 + (E_1 - \alpha \cdot \frac{N_n}{N_{\text{max}}}))^n \quad (4) \]

Although there is no simple solution for this formula, it can be written as followed:

\[ N_n = N_0 + \sum_{i=1}^{n} N_{i-1} \cdot (E_1 - \alpha \cdot \frac{N_{i-1}}{N_{\text{max}}}) \quad (5) \]

The influence of the efficiency and the variation of the efficiency within a PCR are shown graphically in Figure 1. This shows that the quantity of PCR product at the end of a PCR experiment depends not only on the initial concentration of the target (\( N_0 \)), but is also strongly influenced by unknown variables which can not be controlled (e.g. \( E \) and \( \alpha \)) [3,4].
Figure 1. PCR product formation, calculated with different equations. The following values are used $N_0 = 5$, $E = E_1 = 0.7$, $\alpha = 1$ and $N_{\text{max}} = 10^8$.

![Influence of start copy number in PCR model 5](image)

Figure 2. The theoretical PCR product formation, calculated using Equation 5. The following values are used; $E_1 = 0.7$, $\alpha = 1$, $N_{\text{max}} = 10^8$ and $N_0$ is 1, 5, 50, 500 or 5000 copies.

In A and B, the same values are presented, plotted on a logarithmic Y-axis in A, and on a linear Y-axis in B.
Absolute Quantification versus Relative Quantification

Within quantitative PCR, the terms absolute and relative quantification should be distinguished. For example, in GMO analysis, quantification methods have the following differences:

- **Absolute quantification**: The amount of transgenic DNA in a sample is quantified in mass of DNA per volume DNA extract (e.g. ng/µl).

- **Relative quantification**: The amount of transgenic material in a sample is given as a percentage of the total material, e.g., the percentage of transgenic corn in the corn flour of a biscuit. For a relative quantification, an endogenous reference gene is used as a standard.

For a sample which contains only a single material matrix (e.g. only soybean or corn), absolute quantification data can be used to calculate relative amounts. Therefore the transgenic DNA concentration is related to total DNA.

Strengths, Weaknesses and Limitations

The main advantage of PCR is the exponential character of the reaction. This makes it possible to amplify as little as one copy of a target molecule to a detectable concentration.

The major disadvantage is its sensitivity toward small changes in the reaction conditions. Therefore minor changes can have a major influence on the reaction, especially in the later phase where the reaction rate is decreasing. The use of PCR as a quantitative reaction is therefore limited to the phase of the reaction where those minor changes have little influence on the reaction rate.

Overview of General Methods

In general, three types of quantitative PCR can be distinguished: PCR-ELISA with the detection of PCR products in the exponential phase, Quantitative Competitive PCR (QC-PCR), and Real-time PCR.
**PCR-ELISA**

**Method Specification**

In PCR-ELISA [8,9], the amplification of a PCR is linked to the very sensitive detection of the products by an Enzyme Linked ImmunoSorbent Assay (ELISA) and is based on the following principles:

- A reduced number of PCR cycles is used to avoid the plateau phase (see Figure 3);
- PCR products are quantified with a very sensitive ELISA, which has a large dynamic range (see Figure 4);
- A logarithmic relation between initial number of target copies in the PCR and the ELISA signal can be obtained;
- The method can be calibrated with standards for quantitative use.

**Strengths, Weaknesses and Limitations**

**Strengths:**
- Linear dynamic range of 2 to 5 orders;
- Relative quantification is possible with an additional endogenous gene quantification;
- Suitable for high throughput and routine analysis;
- Suitable for automation;
- Highly specific (probe hybridization).

**Weaknesses and Limitations:**
- Due to the reduced number of PCR cycles the method will have a higher lower limit of detection (LLD) and lower limit of quantification (LLQ);
- Although a linear dynamic range as large as 5 orders is reported, in general the linear dynamic range will be limited. The LLQ depends on the lowest target molecule concentration able to generate a reproducible quantitative signal. The upper limit of quantification depends on the highest concentration at which the PCR remains below the plateau phase with the number of cycles applied in the analysis;
- Can be sensitive to small uncontrollable variations;
- Few applications reported in literature;
- ELISA plate reader needed.
Figure 3. A modulation of how the number of PCR cycles will influence the relation between start copy number and quantity of PCR products. The model is represented by Equation 5 with the following parameters: $E_1 = 0.6$, $\alpha = 1$, $N_{\text{max}} = 10^8$ and $N_0$ is 5, 50, 500 or 5000.

A. The product formation during the PCR with different start copy numbers.

B. The quantity of PCR products versus the start copy number after 10, 20 or 30 cycles. Only with a reduced number of PCR cycles (e.g. $n = 25$) is the logarithmic relation between start copy number ($N_0$) and quantity of amplification product ($N$) preserved.
Figure 4. Four principles of the ELISA methods for the detection of PCR products. In general the following steps can be distinguished:
A. The fixation of the PCR product or probe to a solid support (e.g. microtiter plate);
B. Hybridisation of PCR product with probe;
C. Binding of antibody-enzyme conjugate to labelled PCR product or probe (e.g. antidigoxigenin-alkaline phosphatase conjugate binding to digoxigenin labelled probe);
D. Colorimetric or chemiluminescence detection of bound antibody-enzyme conjugate (e.g. CSPD®).
Quantitative Competitive PCR (QC-PCR)

Method Specification

In QC-PCR an internal standard is co-amplified with the target DNA [10]. The quantification is based on the following principles:

- An internal standard, homologous to the target will be amplified with the same efficiency in any phase of the PCR, including during the plateau phase (internal standard and target can be amplified with the same primer pair);
- Each sample is amplified with increasing amounts of internal standard, while keeping the sample volume/concentration constant (multiple PCR reactions);
- In the analysis of the PCR products, the internal standard can be distinguished from the target by a small difference in size (20-80 bp), a restriction enzyme site (RE-site) or a hybridisation site;
- The amount of PCR product of the internal standard and target can be quantified;
- The initial internal standard concentration giving the same amount of end products as the target DNA is defined as the equivalence point. This can be determined graphically (see Figure 5);
- At the equivalence point the initial concentration of the internal standard was equal to the initial target concentration (the difference in product size can be taken into account).

Strengths, Weaknesses and Limitations

Strengths:
- The dynamic range can be adapted by changing the internal standard concentrations;
- Can be setup with relatively limited investment costs.
- Many literature references (mainly in mRNA analysis, not in routine analysis).

Weaknesses and Limitations:
- Low specificity compared to PCR-ELISA and Real-time PCR with the Taqman probe;
- Multiple PCRs necessary for one sample (minimum 4 runs). When fewer reactions (less competitor concentrations) are used, or when the competitor concentrations are not chosen around the sample concentration, precise quantification is no longer possible. In those cases the term semi-quantitative might be used;
- Labour intensive (multiple PCRs, gels);
- Not very suitable for high throughput or routine analysis;
- Relative quantification only possible in pure matrix samples (e.g. 100% corn flour, 100% tobacco leaf). In more complex samples a similar analysis with an endogenous gene has to be done, a double QC-PCR or DQC-PCR (very labour intensive);
- A gel image analyser is needed.

The method is based on the assumption that the efficiency (E) of the amplification of the target and competitor are equal. However, even with a high similarity between the sequence for the target and competitor, small differences in efficiency are not unlikely. Due to the exponential character of PCR, those small differences will have a significant influence on the quantification. (An efficiency difference of 0.1 can give a 3.86 fold-difference in product concentration after only 25 cycles).
Figure 5: The different steps of QC-PCR.

A. A sample is amplified with increasing amounts of internal standard. The PCR products are separated on an agarose gel, where the PCR products of the internal standard can be distinguished from the target by a difference in size.

B. With a gel image analyser the quantity of the different PCR products can be determined.

C. The equivalence point can be determined graphically by plotting the logarithm of the ratio standard/target signal versus the logarithm of the standard. The equivalence point is the point where the straight line crosses the log (standard/target) = 0 axis (standard/target = 1, standard = target).

Figure taken from Studer et al. [11].
Other types of Quantitative Competitive PCR

To overcome some of the problems associated with QC-PCR, the technique is linked to several other analytical techniques:

- QC-PCR-ELISA [12,13];
- QC-PCR-HPLC [14];
- QC-PCR-CE (capillary electrophoresis) [15].

Advantages of these methods over conventional QC-PCR are:

- PCR products are no longer analysed on gels;
- Less labour intensive;
- Higher throughput possible;
- More accurate quantification;
- Can be more specific (QC-PCR-ELISA).

Real-time PCR

Method Specification

In real–time PCR [16,17,18], product formation is followed during the whole reaction. Assuming the following principles, the data generated can be used for quantification:

- The number of cycles, necessary to generate a signal significantly higher than the noise, is taken as a quantitative measure and is called cycle threshold (Ct) (See Figure 6);
- At the moment the Ct value is determined, the PCR is still in its exponential phase;
- There is a good logarithmic relation between Ct and the initial amount of target molecules (see Figure 7).

The linear-logarithmic relation between the initial amount of DNA molecules ($N_0$) and the Ct-value can be represented by the following formula:

$$\log (N_0) = a - b \cdot C_t \quad (6)$$

$a$ and $b$ are constant values which can be calculated from the standard curve. Real-time PCR can be quantitative over a linear dynamic range of 5-7 orders.
For each analysis a detection limit is calculated:

\[ \text{Limit} = 10 \times \text{Noise signal} \]

\( C_t \) is the cycle at which the signal exceeds this limit.

**Figure 6:** Real-time PCR data. \( C_t \) values are determined when the PCR is still in the exponential phase (Figure modified from Perkin-Elmer internet site).

**Figure 7:** The linear-logarithmic relation between the \( C_t \)-value and the initial amount of DNA molecules (Figure modified from Perkin-Elmer internet site).
Strengths, Weaknesses and Limitations

Strengths:
- Linear dynamic range of 5 to 7 orders;
- Suitable for high throughput and routine analysis;
- Highly specific (with Taqman probes);
- Relative quantification is possible through quantification of an additional endogenous gene. The ABI PRISM 7700 Sequence Detection System is able to carry out the latter reaction in the same tube as the main reaction by a multiplex Taqman PCR;
- No post-PCR analysis necessary;
- A strongly reduced chance of post-PCR contamination.
- A fast growing amount of applications can be found in the literature.

Weaknesses and Limitations:
- High investment cost in special equipment:
  - ABI PRISM 7700, Perkin-Elmer (approximately 175,000 CHF);
  - ABI PRISM 5700, Perkin-Elmer (approximately 81,000 CHF);
  - Lightcycler, Boehringer-Mannheim (approximately 89,000 CHF);
- Calculations depend upon certain assumptions about efficiency;
- The signal response relationship is linear-logarithmic.

The calculation method for relative quantification as proposed by Perkin-Elmer (the comparative $C_t$ or $\Delta\Delta C_t$ method, [19], is based on the assumption that the efficiency of the reference and target PCR is equal to 1. Although this might be the case for some of the PCRs, in the cases where it is not, major errors are included (see the weaknesses for QC-PCR) [20]. This error can be avoided by using calculation methods not based on assumption $E_{\text{reference}}=E_{\text{target}}=1$ [21];

The linear-logarithmic signal response relationship ($C_t$-values – $N_0$) means that relatively small variations in $C_t$-value will represent relative big variations in the prediction of $N_0$ (With $E=1$ and $C_t=30 \pm 1\%$, the predicted $N_0$ has a variation of approximately $\pm 20\%$). Although it is possible to extract a quantitative measure out of the Real-time PCR data, which has a linear-linear or a log-log relation with the initial amount of target copies, it is still unclear if this can reduce the variation in later cycles.

Overview of Quantitative PCR

Table 1 gives an overview of the strengths and weaknesses/limitations of the three principal quantitative PCR methods. Regarding these factors, Real-time PCR might be considered to be the most advantageous method for quantitative PCR available at the moment. However, the high investment costs might still be an obstacle for many laboratories to start with this technique. Nevertheless, the amount of publications describing the use of Real-time PCR is increasing rapidly as is the number of laboratories offering services in Real-time PCR quantification.
Table 1. Strengths (+) and weaknesses/limitations (-) for the 3 quantitative PCR methods.

<table>
<thead>
<tr>
<th></th>
<th>PCR-ELISA</th>
<th>QC-PCR</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Costs: Development</td>
<td>High (ELISA)</td>
<td>High (competitor)</td>
<td>Medium</td>
</tr>
<tr>
<td>Investment</td>
<td>Medium</td>
<td>+ Low</td>
<td>+ High</td>
</tr>
<tr>
<td>Per analysis</td>
<td>Low-Medium</td>
<td>+ High</td>
<td>- Medium</td>
</tr>
<tr>
<td>Labour intensity</td>
<td>Medium-High</td>
<td>- High</td>
<td>- Low</td>
</tr>
<tr>
<td>Throughput</td>
<td>High</td>
<td>+ Low</td>
<td>- High</td>
</tr>
<tr>
<td>Automation</td>
<td>Possible</td>
<td>+ Difficult</td>
<td>- Possible but not necessary</td>
</tr>
<tr>
<td>Precision</td>
<td>Medium</td>
<td>- Medium</td>
<td>- Medium-High</td>
</tr>
<tr>
<td>Dynamic range</td>
<td>Small-Medium</td>
<td>- Small but can be focused on target concentration</td>
<td>+ Large</td>
</tr>
<tr>
<td>Specificity</td>
<td>High</td>
<td>+ Medium</td>
<td>- High</td>
</tr>
<tr>
<td>Relative quantification</td>
<td>Possible</td>
<td>+ Labor intensive</td>
<td>- Possible</td>
</tr>
</tbody>
</table>

Overview of Tobacco Specific Methods

Although till now, no articles have been published on the quantitative detection of GM-tobacco, several companies are offering this analysis as a service (e.g. [22]). For this analysis a method very similar to the GMO analysis of, for example, soya and maize is used. Of course, for relative quantification a tobacco specific reference gene has to be applied.

Both QC-PCR and real time PCR are used as (semi)-quantitative PCR methods for the detection of GM plant material [11,22,23]. The QC-PCR method described by Studer et al [11] was validated and successfully tested in an inter-laboratory test in Switzerland and a similar ring test has been launched for the EU. The method will reportedly be the recommended method for labeling compliance according to revised Food Ordinance in Switzerland. However, due to the fast development in the area of quantitative GMO detection, it might be that this method will not be imposed as the only one which can be used. Due to the advantages of Real-time PCR over QC-PCR and the rapidly increasing number of labs offering Real-time PCR GMO quantification, it is likely that in practice Real-time PCR will become the most used technique for quantitative GMO analysis.

Recently, scientists at Philip Morris Europe R&D supervised the development of a method for the quantitative detection of GM-tobacco material in tobacco leaf using Real-time quantitative PCR. The single copy gene nitrate-reductase (*Nia*) was chosen as the reference gene for relative quantification. In order to overcome the weaknesses of Real-time PCR, specific data treatment and calculation methods are under development. The method has been successfully tested for the analysis of large sample numbers. Those analyses showed that with Real-time PCR, the PCR analysis is no longer the limiting step in quantitative GMO analysis.
References


List of Internet Sites

General

PCR Jump Station:  http://www.horizonpress.com/pcr/

Quantitative PCR

PCR-ELISA
http://biochem.boehringer.com/techserv/ttip0498.htm

PCR-Light:
http://www.labsystems.fi/instru/manuals/applicat/an304.htm
http://www.tropix.com/artlantz.htm
http://www.tropix.com/pcrptl.htm
http://www.tropix.com/pcrbak.htm

QC-PCR ELISA
http://biochem.boehringer.com/techserv/ttip0798.htm
http://www.biotecon.com/pcrelisa.htm

Real-time PCR
Perkin Elmer ABI PRISM 7700
http://www.pebio.com/ab/techsupp/7700.html
http://www.pebio.com/ab/about/pcr/sds/realtime.html
http://www2.perkin-elmer.com/ab/about/pcr/sds/wpfig2.html
http://www.wolinsky-lab.cac.nwu.edu/taqman.html

PE-NPTII Taqman
http://www.pebio.com/ag/790601/790601.html

Lightcycler Boehringer Mannheim
http://biochem.boehringer.com/lightcycler/index.htm

Lightcycler manuals (Idaho):
http://www.idahotec.com/pages/user/manuals.html

Lightcycler for the future:
VI. IMMUNOLOGICAL DETECTION METHODS

Introduction

Although genetic modification involves alteration of DNA, the basic chemical from which genes are assembled, genes typically encode proteins. Therefore, for most modifications, it should be possible to detect an altered protein product. Thus, harnessing the vast experience of protein immunoassay as an approach to screening for genetic modification may be advantageous.

The strengths and weaknesses of immunoassay will be outlined and reference will be made to systems that have been used for the detection of proteins derived from genetic modification. The absolute sensitivity and the relative specificity of the methods in question have been sought in all cases because these are key features in developing a screening assay. Robustness of the assay has not been addressed although it would be worthy of consideration for those investigators thinking of developing immunoassay-based screens.

Studies that have directly compared immunodetection methods with DNA-based methods will be considered. The comparison will not be restricted to plant-based systems because the general principles apply to protein immunoassay regardless of the source of the protein. Other authors will consider the use of DNA hybridisation or polymerase chain reaction (PCR) assays as a screen.

Immunoassays have been established for approximately 100 years (e.g. Landsteiner’s haemagglutination assay and the diffusion-immunoprecipitation assays of Mancini and Ouchterlony [1]). However, in this review consideration will be restricted to the more recent developments of enzyme-linked immunoabsorbent assay (ELISA) and its numerous variants.
Reference is often made to the technique of Western blotting, which is a particular form of immunoassay used as a detection system following gel electrophoresis. Although widely used in laboratory investigations, this technique has little applicability for general screening and it will not be considered further.

All of these assay techniques are discussed in more detail in Reference 1.

Outline of Strategies

There is much similarity between an ELISA and a probe-based DNA assay. The antibody in an ELISA recognises a three-dimensional conformation of a structure, whereas a DNA probe recognises specific sequences of DNA. Both rely on complementarity for recognition and high-affinity binding. Further, both ELISAs and DNA probe-based assays rely on non-specific amplification processes to allow detection in an assay system. Antibodies can be raised against DNA; however, because they rely on three-dimensional conformation for specificity, they cannot be truly sequence-specific. A consideration of the possibility of using anti-DNA antibodies to detect genetic modification in tobacco is outside the scope of this review. More often, antibodies are raised against protein, lipoprotein or carbohydrate residues and all have been used in ELISA.

The ELISA is extremely flexible and it is used in many forms. The basic assay system is quantitative, comparing the test material against a known standard material. All of the assay variants rely on similar principles, and these will be outlined in the next section.

The strategies that could be used for designing a screen for potential genetic modification of tobacco using ELISA are similar to those used in PCR-based screens. However, given the longer lead-time for the establishment of an ELISA, discussed below, it is likely that any use of such systems will be restricted to the available assays for selectable markers. In theory, the flexibility of ELISA would allow an assay to detect a molecule that would only be present as a result of genetic modification, in a direct analogy to using a DNA probe that spans through a DNA sequence from, for example, viral promoter to plant protein gene. In an ELISA this would be achieved by using antibodies of differing specificity at the capture and detection steps (described below), in a system known as a double determinant assay. Note, however, that each specific assay would require a unique antibody at one of the steps and so this is unlikely to be a realistic choice for screening or confirmation of other positive results.

Overview of General Methods

The most widely used and most flexible type of ELISA is known as a sandwich assay in which antibodies are used to capture and detect the material of interest. However, other variants do exist. A sandwich ELISA can be broken into two steps, capture of the material of interest and generation of an amplified signal with a detection system. The antibodies used in these separate steps may be monoclonal or polyclonal and they need not have identical specificity. In the capture step the antibody is fixed unto a solid surface, typically a plastic microtitre plate but it may be fixed to paper or other fibrous material or to a microbead. The surface bound antibody is exposed to a solution containing the test material or a known standard. In variations of the ELISA, a known amount of labelled standard material may be introduced at this stage.
After washing away unbound material, a detection antibody system is applied. The detection antibody specificity may be identical, similar or completely different to the capture antibody. For detection, the antibody will have been chemically coupled to an enzyme, for example alkaline phosphatase or horseradish peroxidase. Again, after washing away any unbound detecting antibody, enzyme substrate is added to yield typically a coloured product, in proportion to the amount of enzyme bound. Thus, the colour generated is proportional to the amount of unknown or standard bound to the solid-phase capture antibody. For plate-based assays, the colour generated by this reaction is detected on an automatic spectrophotometer and the results are available within minutes.

In a potential screen for genetically modified plants, a capture antibody that detects one domain of a protein could be used in a system with a detection antibody with specificity for another domain of interest. This second domain could be one that would only be expected if a particular genetic modification were expressed. For example, a viral domain expressed in a plant protein would suggest genetic modification, if the virus from which the protein domain was isolated was known not to infect the plant genus in question.

### Absolute Sensitivity

Numerous authors appear to have defined a similar limit of sensitivity for ELISA systems and some examples are given in Table 1 below:

<table>
<thead>
<tr>
<th>System</th>
<th>Reported Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhizae Radix Protein</td>
<td>100 pg [2]</td>
</tr>
<tr>
<td>Alfalfa Mosaic Virus Protein</td>
<td>1 ng/ml [3]</td>
</tr>
<tr>
<td>Human Immunoglobulin</td>
<td>1 ng/ml [4]</td>
</tr>
<tr>
<td>UV-irradiated DNA</td>
<td>1 pg [5]</td>
</tr>
</tbody>
</table>

In the examples above an absolute limit of sensitivity of approximately 500 pg/ml (which is 100 pg total added material, for a reaction volume of 200 microlitre) was reported. For the example of an anti-DNA antibody detection system [5], an absolute detection level of approximately 1 pg was claimed, although this does not reflect the typical experience in other reported examples. Nonetheless, it appears that the lower limit of sensitivity for immunoassay detection systems is in the picogram range.

### Comparative Sensitivity

Several investigators have compared directly the sensitivity of antibody-based and DNA-based detection in the same study. The four examples shown in Table 2 are all taken from studies using human clinical samples.
Table 2. Comparison of sensitivity of PCR and Immunoassay detection systems.

<table>
<thead>
<tr>
<th>Screen Target</th>
<th>PCR Sensitivity (%)</th>
<th>Immunoassay Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes simplex virus</td>
<td>59</td>
<td>39</td>
</tr>
<tr>
<td>Chlamydia [7]</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>Rotavirus [8]</td>
<td>92</td>
<td>73</td>
</tr>
<tr>
<td>Chlamydia [9]</td>
<td>88</td>
<td>81</td>
</tr>
</tbody>
</table>

In these studies patients known to be at high-risk for the appropriate infection were screened using a battery of test regimes. By comparing the sensitivity of ELISA with, amongst others, PCR-based assays, the PCR approach was reported to be the most sensitive.

Relative Specificity

The relative specificity of a system is harder to define and often depends on how carefully that the investigator examines the system in question. In the four studies of human clinical material cited above [6,7,8,9], the investigators all quoted relative and sometimes absolute specificity for the various detection methods. However, in all cases, PCR analysis was the most sensitive and, therefore, other assay systems were assumed to be giving a proportion of false negative results. Although confirmation of positives was sought in more than one assay system, the possibility of false positive results cannot be overlooked. One group commented, “Isolation by cell culture has been considered as the ‘gold standard’ for detection of HSV in genital lesions, but in this study HSV PCR was significantly more sensitive” [6]. Thus, the apparently superior sensitivity of PCR-based assays may arise from decreased relative specificity, due to false positive results.

Other Considerations

Other features of ELISA systems may generate advantages and disadvantages for screening, in comparison with DNA-based systems. A major advantage is that detection of a protein or carbohydrate epitope using an immunoassay system is a guarantee that a gene product has been expressed. This cannot be assumed for a DNA probe-based assay. Conversely, a disadvantage of immunoassays is that any genetic modification that only leads to alterations in DNA will not be detected by immunoassay.

The apparent lower sensitivity of the immunoassay systems is not an automatic disadvantage because a false positive result from a minor contaminant is less likely. Further, immunoassay systems are all quantitative, with limits of detection that are sufficient for most screening applications.

The major disadvantage of a screen based on an immunoassay system is experienced at the outset. The time to generate a specific antibody against the antigen of concern can be great (six months is not uncommon). Once an appropriate (monoclonal or polyclonal) antibody is generated, careful standardisation and testing for unexpected cross-reactivity must be performed. Because an antibody detects a three-dimensional conformation, unexpected false positives do occur rarely. Nonetheless, once established, the flexibility and turn-around time
for immunoassay systems is excellent. With 96 well microtitre plates and automatic detection systems, a complete assay can be performed in two to four hours and a single operator can perform a large numbers of screens.

**Overview of Tobacco Specific Methods**

The general principles described above have been used in systems to detect tobacco-related proteins; however, no unique features have been reported for tobacco. Further, no specific assays for the detection of genetically modified tobacco have been reported.

As noted above, ELISA systems with specificity for marker genes widely used in plant genetic modification have been reported. Rogan et al [10] described the sensitivity, accuracy and precision of an ELISA system developed to quantitate the levels of neomycin phosphotransferase II (NPT II) in genetically modified cotton seed and leaf tissue. Their ELISA system could be used for any plant-derived material. A kit assay system for NPT II is available in a variety of sizes from at least one supplier [11].

Other commercial ELISA kits for a variety of applications are becoming available. Of interest are kits that are reported to detect specific proteins in food crops. One company offers an ELISA plate-based kit to detect a specific protein in foodstuffs derived from genetically modified soybeans and a “dipstick” field testing procedure for crop and seed monitoring [12]. The dipstick test is claimed to run in approximately 20 minutes and uses an immunochemical method of detection; thus it may be regarded as a variant of the ELISA systems described above. The plate-based kit is reported to cost an average of Euro 14.50 per sample.

Recently, the Food Products Unit of the Institute for Health and Consumer Protection (IHCP, Joint Research Centre, European Union Commission, Ispra, Italy) has determined the performance of the diagnostic ELISA kit from Strategic Diagnostics Inc., mentioned above. This was a collaborative trial to detect and quantify genetically modified "Roundup-Ready®" soybean, involving the Reference Material Unit of the Institute of Reference Materials and Measurements (IRMM) and 38 laboratories from 13 EU Member States and Switzerland. In this validation study (also see Section VIII) the ELISA assay gave an incorrect assignment of GM status in only 1% of samples in which the GMO was present at a level of 2% or greater. (See: http://www.jrc.org/jrc/index.asp and http://www.sdix.com/).

**Summary**

The most widely used form of immunoassay currently in use is the ELISA. This quantitative assay is extremely flexible, with a limit of detection typically in the 500 pg/ml range. Where direct comparisons have been made, PCR is reported to be more sensitive than ELISA. However, in these direct comparisons it remains open to question whether this superior PCR sensitivity is achieved due to a loss of specificity, with the generation of false positive signals. When assay turn-around time is considered, ELISA appears to offer some advantage; however, the establishment of an ELISA for a novel unknown material is more complex than for a PCR assay. For ELISA, the lead-time required may be six months or greater. On a simple cost basis, there may be little to choose between the systems but generally, the equipment costs are lower for ELISA. Field variants of the ELISA, such as dipstick kits, have been developed and they offer a semi-quantitative test of some practical value.
References


11. 5 Prime → 3 Prime, Inc, 5603 Arapahoe Road, Boulder CO 80303, USA.

VII. SELECTABLE GENETIC MARKER-BASED DETECTION METHODS

Introduction - The Importance of Genetic Markers in Plant Transformation

One of the most important advances in plant vector construction in particular, and in gene transfer to plant cells in general, was the development of genetic markers applicable in plant tissue. These bring about a phenotypic change in the cell which demonstrates that foreign DNA has entered the cell, that it is being expressed, and also being passed on to progeny. Moreover, the development of dominant selectable markers functional in plant tissue allows the direct selection of transgenic cells by their ability to grow and proliferation under selective conditions.
Outline of Detection Strategies

Detection strategies can be based upon identifying the presence of selectable marker genes in transgenic plants. Methods for identifying selectable marker genes in seeds or plantlets may be direct (e.g. assessing the impact of a selective agent in seedling tests), or indirect (e.g. enzymatic assay, bacterial complementation).

Overview of General Detection Methods

The general approach to detecting marker genes in plants is irrespective of the genes in question. A selection of genes encoding the most commonly-used selectable markers is given below, along with examples of specific detection methods.

Selectable Marker Genes

Neomycin phosphotransferase

Neomycin phosphotransferase (nptII, neo), encoded by the bacterial transposon Tn5 [1] (Genbank : JO1834), confers resistance to aminoglycoside antibiotics and has become an important marker for use in plant cells largely because selection for resistance can be applied to isolated cells, callus, tissue explants and whole plants [2,3].

Hygromycin phosphotransferase

Hygromycin B is another aminoglycoside antibiotic which disrupts ribosome function in a variety of cell types. Resistance in E. coli is encoded by an hygromycin phosphotransferase gene (aphIV) [4] (Genbank : VO1499), which, when transferred to tobacco cells, confers resistance to levels of hygromycin of up to 50 mg/l [5].

Streptomycin phosphotransferase

The streptomycin resistance gene, encoding streptomycin phosphotransferase (spt) and derived from Tn5 [6], (Genbank : L19385), has been engineered into tobacco and confers resistance to streptomycin at levels of 1 mg/ml [7].
**Dihydrofolate reductase**

Dihydrofolate reductase (DHFR), involved in the conversion of dihydrofolate to tetrahydrofolate, is inhibited by methotrexate which results in impaired RNA and DNA synthesis. A gene has been derived from *E. coli* that has been found to be insensitive to methotrexate [8], (Genbank : X00926), and in an appropriate construct confers resistance to methotrexate in plant cells [3].

**Enolpyruvylshikimate-3-phosphate synthase**

Glyphosate inhibits enolpyruvylshikimate-3-phosphate synthase (EPSPS) which, being involved in the shikimate pathway, is important in the synthesis of aromatic amino acids. Tolerance to glyphosate can be obtained by over-production of EPSPS encoded by the aroA gene [9] (Genbank : X63374). Selection can be applied either at the callus stage (0.5mM glyphosate), or by spraying regenerated plants. The latter are tolerant to 2-4 times the amount of glyphosate required to kill wild type plants.

**Phosphinotricin acetyltransferase**

The bialaphos (phosphinotricin or PPT) resistance gene (*bar*) from *S. hygroscopicus* (Genbank : A02804) encodes phosphinotricin acetyltransferase (PAT). This enzyme acetylates a free amino group of phosphinotricin and detoxifies it [10]. The gene provides resistance of up to 50 mg/l of PPT in calli and whole tobacco [11].

**Nitrilase**

The *bxn* gene from *Klebsiella pneumoniae* (Genbank : J03196) encodes nitrilase which metabolizes oxynil herbicides [12].

**Acetolactate synthase**

The acetolactate synthase (*als*) gene from tobacco provides resistance to sulfonylurea herbicide [13].
Direct Detection Method

Impact of a selective agent (seedling tests)

Surface-sterilised seeds are placed on to the surface of solidified medium with or without the selective agent present. Bleaching or greening is scored after 2 weeks of culture. Alternatively, a chlorophyll fluorescence assay can be used to screen the plants’ resistant to antibiotics [14] or herbicides [15].

Table 1. Concentrations of the selective agent used for seedling tests.

<table>
<thead>
<tr>
<th>Selective Agent</th>
<th>Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>170 [2]</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>100 [3]</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>340 [7]</td>
</tr>
<tr>
<td>Bromoxynil</td>
<td>100</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>100</td>
</tr>
<tr>
<td>Chlorsulfuron</td>
<td>50 [16]</td>
</tr>
</tbody>
</table>

(Assays are normally conducted in 10cm Petri dishes containing 20ml solidified MS medium [17] (7g/l agar), under a 18h photoperiod at 25°C [18]).

Indirect Detection Methods

Enzymatic assay of NPTII

This enzyme assay involves the electrophoretic separation of a cell extract on a non-denaturing polyacrylamide gel [19]. The gel is then equilibrated against the reaction buffer, following which an agarose gel containing (γ32P)-ATP and kanamycin sulphate is cast to the non-denaturing gel. The enzyme activity produces phosphorylated kanamycin which is transferred to paper by blotting and can be visualized by autoradiography.

This method has the disadvantage of using very high levels of radioactivity.
**Bacterial complementation**

This assay is based on the recombinational repair of a mutant *nptII* gene [20]. When competent *Acinobacter* sp. BD413 cells containing a *nptII* gene with an internal 10bp deletion are transformed with DNA carrying a functional *nptII* gene, kanamycin-resistant transformants are obtained. When transformed with total DNA from a GM plant, the number of kanamycin resistant transformants is in proportion to the concentration of *nptII* genes in the plant DNA.

Detection of *nptII* by bacterial complementation is 500- to 1000-fold less sensitive than PCR.
**Overview of Tobacco Specific Methods**

The list of tobacco plants which have been field tested in the US reveals the present situation with respect to the use of the most common selectable markers in tobacco.

**Table 2.** APHIS Field tests permits and notifications 1987-1997.

<table>
<thead>
<tr>
<th>Marker (%)</th>
<th>nptII (65)</th>
<th>DHFR (1)</th>
<th>EPSPS (1)</th>
<th>PAT (3)</th>
<th>bxn (2)</th>
<th>als (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notifications (%) (89)</td>
<td>(1.4)</td>
<td>(1.4)</td>
<td>(4.1)</td>
<td>(2.7)</td>
<td>(2.7)</td>
<td></td>
</tr>
</tbody>
</table>

No notifications were made using the *aphII* or *spt* genes

Both the direct and indirect detection methods described above could be applied to green tobacco leaf, but it is unlikely whether any of the tests could be applied successfully to cured leaf, with the possible exception of the bacterial complementation assay.

**Summary**

The detection of GMO based on selectable markers genes may be problematic in the near future because some protocols proposed recently aim to eliminate these types of genes from the transformation process [21,22]. At present these methods of detection are less sensitive than PCR-based methods and are more difficult to apply to cured leaf.
References


VIII. OFFICIAL AND VALIDATED METHODS

Introduction............................................................................................................................ 73
European Union Validated Methods...................................................................................... 74
  Screening Method for the Identification of GMOs in Food: ............................................. 74
  Methods Under Evaluation and Validation................................................................. 76
    Detection of Genetically Modified Soybeans........................................................... 76
    Quantitative Competitive PCR (QC PCR)............................................................... 77
GMO Reference Materials.................................................................................................. 78
Official Methods – Germany............................................................................................... 78
  Official GMO Detection Methods............................................................................... 78
    Detection of Genetically Modified Soybeans........................................................... 78
    Detection of Genetically Modified Potato.............................................................. 79
    Detection of Genetically Modified Lactobacillus curvatus in Raw Sausage-Meat...... 79
    Detection of Genetically Modified Streptococcus thermophilus in Yogurt.............. 79
  Method Published by BgVV....................................................................................... 79
  Detection of Genetic Modification of Plants............................................................... 79
Methods in Preparation..................................................................................................... 80
Official Methods – Switzerland.......................................................................................... 80
  Screening Method for the Identification of GMOs in Food.............................................. 80
  Method Under Evaluation ......................................................................................... 81
Activities Of Trade Organizations.................................................................................... 82
GMO Analysis Laboratory Accreditation........................................................................... 83
Conclusions....................................................................................................................... 83
Summary............................................................................................................................ 84
References......................................................................................................................... 86
List of Internet Sites............................................................................................................ 87

Introduction

Biotechnology regulations require premarket risk assessment of genetically modified (GM) food crops and provide mechanisms for approving GM foods and ingredients shown to be substantially equivalent to their conventional counterpart. However, the issues associated with the labeling and traceability of genetically modified organisms (GMOs) have not been clarified. The techniques used to detect a genetic modification reveal either the specific DNA sequence introduced into the plant or the product (RNA or protein) generated by the genetic modification. In particular, the polymerase chain reaction (PCR) technology and hybridisation techniques reveal the DNA or RNA sequence, whereas antibody probes and enzymatic activity tests detect the presence of proteins (for a recent review on GMO detection methods see [1]). PCR is currently the technology of choice for the detection of genetic modifications in plants.
The adoption of official or validated methods as a tool for detecting GMOs in raw agricultural materials and finished products is in its initial stage. Germany and Switzerland are currently the only countries having official methods whereas the European Union (EU) has recently validated a screening method.

This section reviews the current status of the official and validated methods that are applicable or are under development for the detection of GM raw agricultural crops and/or finished products.

**European Union Validated Methods**

According to the Novel Food Regulation EC 258(98) and Council Regulation EC 1139(98) (labeling of two particular GM soybean and corn products) of the European Union (EU), foods have to be labeled if modified DNA or newly expressed proteins can be detected and their content exceeds a threshold amount (a *de minimis* 1% threshold has been recently proposed). However, the EC 1139 regulation does not specify test methods to be used, method validation, or criteria for selecting a method for different applications.

The Joint Research Centre (JRC, Ispra, Italy) of the European Union Commission has set up a GMO method evaluation program to address the above-mentioned issues and has recently validated a "Screening method for the identification of genetically modified organisms (GMO) in food".

The EU method was originally developed by Swiss and German scientists using soybean and corn, the two most common crop plants used for food production. It is based on the detection of DNA fragments that control the activities of the newly introduced genes (the 35S-promoter and the nos-terminator, see Chapter IV of this report). The EU screening method is reportedly capable of detecting the majority of GM agricultural products presently approved in the EU (26 out of the 28 GMOs already approved or being considered for approval can be detected using this method).

The following is a brief description of the method.

**Screening method for the identification of genetically modified organisms (GMO) in food** –

Detection of the CAMV 35S promoter and nos terminator by means of the polymerase chain reaction (PCR) [2].

The method was issued by E. Anklam and M. Lipp of the EU JRC (in collaboration with P. Brodmann, Kantonales Laboratorium Basel-Stadt, Switzerland and K. Pietsch, Chemische Landesuntersuchunganstalt, Freiburg, Germany) and is based on the published method for the detection of the genetic modification markers 35S promoter and nos terminator [3]. It has become an official Swiss method [4] and the same principle of the method is also the basis for the official German method [5].
It should be noted that this method has been validated for the qualitative detection of GM raw agricultural products and is not suited to detect the presence of GMOs in manufactured food products. Moreover, the detection of the 35S promoter and the nos terminator by the screening method is to be considered only an indication that the analyzed sample may contain DNA from a genetically engineered plant. In case of positive results, further investigation has to be performed in order to detect the introduced gene with more selective tests or methods, and to exclude any false positive result due to possible cauliflower mosaic virus and Agrobacterium tumefacies contamination. (See Section IV, PCR-based Screening Methods).

The main features of the EU method can be summarised as follows:

- DNA is extracted from the sample material by the CTAB method or Wizard-Method, and the yield is assessed by gel electrophoresis;
- the quantity of DNA used for the PCR reaction is 5 - 10 ng (in 100 µl) and the target DNA sequences are part of the 35S promoter and nos terminator respectively;
- the PCR products are separated by electrophoresis and examined by comparison to length standards (PCR amplification results in a fragment of 195 base pairs (bp) for the 35S promoter and a fragment of 180 bp for the nos terminator);
- the presence of the target DNA is confirmed by restriction enzyme analysis (using XmnI for the 35S amplicon and Nsil for the nos amplicon).

The method was validated through a European ring test with twenty-five EN 45000 accredited laboratories from 13 countries (16 governmental organizations, 7 private and 2 university laboratories). The JRC provided the European laboratories with the appropriate reference materials produced at the Institute for Reference Material and Measurements at the JRC (Geel, Belgium).

The validation was performed using reference powders containing Bt-176 corn and Roundup Ready Soya samples. Table 1 summarises the results of the ring test. The results of the validation study demonstrate that the EU screening method is suitable for the detection of GMO in raw material derived from soybean and corn having a GM concentration of 0.5% or higher [6]. Samples containing 2% of GMO (both soybean and corn) were always correctly identified by all the laboratories, thus demonstrating the validity of the method for screening purposes (for a review of the ring test, see [7], and October 6, 1998 press release of the EC Joint Research Center in http://www.jrc.org/jrc/index.asp).

Table 1
Analysis of maize for CaMV 35s promoter

<table>
<thead>
<tr>
<th>% GMO in Matrix</th>
<th>Correct Classification</th>
<th>Incorrect Classification</th>
<th>Relative Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>88</td>
<td>1</td>
<td>1.1 %</td>
</tr>
<tr>
<td>0.1 %</td>
<td>74</td>
<td>14</td>
<td>15.9 %</td>
</tr>
<tr>
<td>0.5 %</td>
<td>83</td>
<td>3</td>
<td>3.5 %</td>
</tr>
<tr>
<td>2 %</td>
<td>89</td>
<td>0</td>
<td>0 %</td>
</tr>
</tbody>
</table>
Analysis of soybean powder for CaMV 35S promoter

<table>
<thead>
<tr>
<th>% GMO in Matrix</th>
<th>Correct Classification</th>
<th>Incorrect Classification</th>
<th>Relative Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>94</td>
<td>2</td>
<td>2.1 %</td>
</tr>
<tr>
<td>0.1 %</td>
<td>93</td>
<td>5</td>
<td>5.1 %</td>
</tr>
<tr>
<td>0.5 %</td>
<td>105</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>2 %</td>
<td>101</td>
<td>0</td>
<td>0 %</td>
</tr>
</tbody>
</table>

Analysis of soy bean powder for nos terminator

<table>
<thead>
<tr>
<th>% GMO in Matrix</th>
<th>Correct Classification</th>
<th>Incorrect Classification</th>
<th>Relative Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>96</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>0.1 %</td>
<td>91</td>
<td>7</td>
<td>7.1 %</td>
</tr>
<tr>
<td>0.5 %</td>
<td>102</td>
<td>3</td>
<td>2.9 %</td>
</tr>
<tr>
<td>2 %</td>
<td>101</td>
<td>0</td>
<td>0 %</td>
</tr>
</tbody>
</table>

EU Methods Under Evaluation and Validation

Following the guidelines adopted by the Commission for the 5th Framework Program, the JRC is pursuing efforts to improve GMO analysis methods within a broader program. This is divided into fifteen work packages and covers thirteen foods or products. Crucial points are the DNA-extraction of raw and processed foods, and the development and testing of primers and probes for PCR systems.

In parallel to the straightforward development of methods based on PCR and hybridization procedures, other methods are investigated which have the potential to increase the efficiency of the analysis performed routinely. Such methods include detection of marker genes, development of multiplex primer systems, microtitre plate based sandwich-type DNA probe assay (PCR-ELISA), and DNA biosensors. Further methods are undergoing testing which might support or substitute PCR/Hybridization procedures such as direct hybridization, isothermal nucleic acid sequence based amplification (NASBA), selfsustained sequence replication (3SR) techniques for actively transcribed modified sequences, AFLP fingerprinting and protein diagnostic methods.

In particular, the EU Joint Research Center is in the process of finalizing the evaluation of two methods, e.g. Detection of the CP4 EPSPS protein present in Roundup Ready Soybeans (RRS), and Quantitative-Competitive PCR (QC PCR).

Detection of Genetically Modified Soybean protein

This is an immunochemical method for the detection of the CP4 EPSPS protein present in Roundup Ready® Soybeans (RRS), developed by Strategic Diagnostics Inc. (SDI, Newark, Delaware; see also Chapter VI of this report). The test kits are based on an ELISA sandwich
assay, using the principle that an antibody bound to a microtitre plate well also binds to a new protein in the sample and can demonstrate its presence. The new protein's presence is visualized by binding yet another antibody to the sample in a sandwich fashion. The second antibody has an enzyme tag that can produce a colour reaction. If there is any genetically modified protein present, the color production can be quantified using a spectrophotometer. The advantages and disadvantages of this semi-quantitative method are discussed in Section VI of this report.

SDI recently announced that its Soya Test Kits have been validated as a test for compliance with the food labeling regulations within the EU member countries. The EU Joint Research Center, together with the Reference Material Unit of the Institute of Reference Materials and Measurements (IRMM) evaluated, in a collaborative trial involving 38 laboratories from 13 EU Member States and Switzerland, the performance of the diagnostic kit to determine the relative amount of GMO present in defined mixtures of finely ground soybean powder. The table below summarizes the results in terms of samples classified as positive (equal or greater than the threshold of 2%) and samples classified as negative (below the threshold of 2%).

<table>
<thead>
<tr>
<th>% GMO in sample</th>
<th>Samples classified as positive</th>
<th>Samples classified as negative</th>
<th>% of wrong classification (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>148</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>148</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>147</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>140</td>
<td>1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

(*) Statistical data interpretation revealed that with 99% confidence any sample scoring as negative contains less than 2% GMO and any positive sample contains at least 0.85% GMO.
(Source of data: JRC-IHCP)

Quantitative Competitive PCR (QC PCR)

The EU Standing Committee for Food has recently given a favorable opinion on a Commission draft regulation that would introduce a *de minimis* GMO threshold of 1%. The threshold takes into account the possibility of presence of GMO DNA or protein traces from accidental contamination (i.e., evidence must be provided that efforts to avoid GMO sources have been undertaken). However, it will only apply to EU-authorized GMOs and to individual primary ingredients (e.g., flour, starch, syrup, etc.)
The determination of compliance with the labeling provisions and threshold may be carried out with the quantitative competitive PCR technique (reviewed in Chapter V of this report and [8]).

**Official Methods - Germany**

The Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV - Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany; http://www.bgvv.de/) has coordinated since 1994 a working group on the development of the official GMO analysis methods. After ring trials and validation, the methods are included in the “Collection of Official Methods Under Article 35 of the German Federal Foodstuffs Act (LMBG – Lebensmittel und Bedarfgegenständegesetz).

**German Official Methods for GMO Detection**

(LMBG published and also published in BgVV–Hefte [9])

The methods published in the LMBG are specific to a gene or GM-crop, and include a description of procedures for DNA extraction, PCR analysis and a confirmatory test:

- DNA from the sample is extracted according to a specific procedure and the yield is checked by gel electrophoresis;
- the target DNA sequence is amplified by means of a suitable primer in a PCR and the amplification capacity of the extracted DNA is checked in a parallel replicate with a primer pair derived from a region of the plant genome;
- the PCR products are then separated by agarose gel electrophoresis and examined for the expected product sizes.

In addition, the sequence of the amplified DNA fragment of the genetically modified plant is confirmed by a DNA hybridization assay with a labeled DNA probe following its transfer from the gel to a membrane (Southern blot). The sequence of the DNA probe must be complementary to a sequence region lying between the sequences of the primer pair used.

**Detection of Genetically Modified Soybeans**

In this screening method [10], the genetic modification is detected as specific DNA sequences encoding the CaMV 35S promoter sequence linked to the *Petunia hybrida* chloroplast transit-signal sequence. Confirmation is performed by hybridizing a labeled 35S promoter sequence to the amplified product. The amplification capacity is checked with primers specific to the lectin gene. Jankiewicz et al. of BgVV have recently published a study on the theoretical and practical detection limits in GM crops of the screening method, which were reported to be 0.005 % and 0.1 % GMO/non-GMO (w/w), respectively [14].
Detection of Genetically Modified Potato

This method [11] detects specific DNA sequences encoding the hygromycin phosphotransferase gene. Hybridization using a labeled hygromycin phosphotransferase sequence is used as confirmation. Amplification suitability is checked in this case using primers specific to a chloroplast tRNA sequence.

Detection of Genetically Modified *Lactobacillus curvatus* in Raw Sausage-Meat

Sequences encoding the catalase-gene (*katA*-gene) and the chloramphenicolacetyltransferase gene (*cat*-gene) are detected by this method [12]. Hybridizing a labeled catalase gene sequence to the amplified product is used for confirmation. Primers specific primers to the conserved regions of the 16S-rDNA gene of eubacteria are used to confirm amplification capacity.

Detection of Genetically Modified *Streptococcus thermophilus* in Yogurt

By this method [13], sequences encoding the chloramphenicolacetyltransferase gene (*cat*-gene) and *lacZ* gene are detected, with confirmation by hybridizing a labeled *cat*-gene and *lacZ* gene junction sequence to the amplified product. The amplification capacity is checked using primers to the *lacZ* gene of *Streptococcus thermophilus*.

Method Published by BgVV

Detection of Genetic Modification of Plants

(Screening procedure for the identification of genetically modified DNA sequences in plant with the detection of DNA-sequences that occur often in genetic modified organism (Screeningverfahren zum Nachweis gentechnisch veränderter DNA-sequenzen in Pflanzen durch den Nachweis von DNA-Sequenzen, die häufig in gentechnisch veränderten Organismen vorkommen) [5])

The method is similar to the EU-validated and Swiss official methods:

- DNA is extracted from the sample material using the CTAB-method, and the yield of DNA is assessed by gel electrophoresis;
- 10 – 50 ng DNA is used in the PCR reaction (25 µl);
- the target DNA sequences are part of the 35S promoter and nos terminator;
- the PCR products are separated by electrophoresis and products of the expected sizes are sought;
- the presence of the target DNA is confirmed by restriction enzyme analysis.
German Methods in Preparation

Methods to be published in 1999 include the detection of genetic modification in tomato (e.g. Zeneca's tomato), and a Quantitative-Competitive PCR method.

Official Methods – Switzerland

The Swiss Ordinance on Food Stuffs of 1995 (LMV) and its 1999 amendments [15,16] have established that all foodstuffs, food additives and processing aids consisting of/or derived from GMOs must have premarket approval and have to be labeled. Accordingly, the Swiss Federal Office of Public Health has published an official method for the screening of genetically modified organisms (GMO) in food in the Swiss Food Compendium 52B: Methods in Molecular Biology [4]. The PCR method is derived from Pietsch et al. [3], whereby the detection of the 35S promoter and/or nos-terminator indicates the presence of a genetically modified plant and triggers GMO labeling. Positive samples have to be confirmed with specific methods according to the example described in "Method 52B/4: Detection procedure for genetically modified Roundup Ready™ soybeans (RRS) in foods".

Swiss Screening Method for the Identification of GMOs in Food

Detection of the CAMV 35S-promoter and nos-terminator by means of the polymerase chain reaction (PCR) [4]

This method is similar to the EU validated method (a variant of the general method is stipulated for additives such as lecithin):

- DNA is isolated from the sample using the Wizard®-method [http://www.promega.com/techserv/default.htm] and assessed either by gel electrophoresis or photometrically;
- the quantity of DNA used in the 100 µl PCR reaction is up to 500 ng;
- the PCR targets, products and product confirmation procedures are as described earlier.

The sensitivity of the method is such that 1 ng of pure RRS DNA or 10 fg of linearized pBI121 plasmid DNA gives a positive signal (this corresponds to approximately 600 genome copies in the reaction).

Hübner and colleagues have recently reviewed the critical points in the quality assurance of the Swiss screening method [17] and recommended a set of guidelines to improve laboratory performance and standardization.
Swiss Methods Under Evaluation

Quantitative Competitive PCR (QC PCR)

An evaluation of quantitative competitive PCR techniques is being performed in order to provide a tool for labeling compliance testing after the recent introduction of a GMO threshold value of 1% for the labeling of foods and foodstuffs [15]. An evaluation of QC-PCR techniques is being performed in order to provide a tool for the introduction of a threshold value of 1% for the labeling of foods and foodstuffs [8]. The threshold was approved in June 1999 and is now enforced through a revised version of the LMV ordinance (Lebensmittelverordnung) [15].

Standardization of GMO Analysis Methods and Availability of GMO Reference Materials

While an increasing number of food control laboratories is adopting PCR as the technology of choice for GMO detection, international standardization and validation of GMO methods by harmonized and accepted protocols is still in its early phases and is currently limited to the EU and Switzerland.

Standardization bodies such as the European Committee for Standardization (CEN, Brussels, Belgium) and the French Standardization Association AFNOR (Paris, France) have recently acknowledged the need for harmonized protocols to validate GMO analysis methods. In particular, CEN has started a harmonization effort in the area of GMO detection methods through its technical committee TC 275 WG11 "Methods for GMO detection". AFNOR has recently created a standardization committee named "Detection of GMO and derived products" (see press release in http://www.afnor.fr), whose activities are linked to CEN. The work program concerns food crops and is carried out in cooperation with service laboratories and agro-food companies under the coordination of the National Institute for Agronomic Research (INRA). It includes the preparation of guidelines on nucleic acid extraction, the definition of qualitative and quantitative methods, and sampling procedures. AFNOR expects to publish experimental norms or documentation on these subjects by the end of 1999. Moreover, discussions are being held at various standardization associations to consider the proposal of involving the International Standards Organization (ISO, Geneva, Switzerland) in the standardization of GMO analysis methods.

Another international body that recently got involved in the standardization and validation of GMO analysis methods is the International Union of Pure and Applied Chemistry (IUPAC, Research Triangle Park, NC, USA; http://www.iupac.org/). The IUPAC Division of Chemistry and the Environment, Commission on Food Chemistry (VI.5) has worked in cooperation with the EU Commission JRC for the validation of the EU screening method [7].

Only a few GMO analysis laboratories have obtained or are in the process of obtaining accreditation: e.g., GeneScan GmbH (Freiburg, Germany) has been accredited in 1998 according to DIN EN 45001 “to check by methods of molecular biology for the presence of specific sequences or structures in plants, their products and viruses”; Reading Scientific Services Ltd. (RSSL, Reading, UK.), which licenses a method from the US company Genetic ID, is currently working toward obtaining accreditation on laboratory performance through the UK Accreditation Service (UKAS).
The development, validation and use of GMO analysis methods ideally demand the availability of certified reference materials. The EU commission Institute for Reference Materials and Measurements (IRMM) has recently produced reference materials for the detection of GM soybean and corn in the form of stable powders containing guaranteed GMO-free flour in pure form and mixed with 0.1 %, 0.5 % and 3.0 % GM flour. The reference materials are commercially available from Fluka. Moreover, it has been proposed that a central repository, perhaps a European Union facility, be created for storage of information (e.g., sequence data, primers, novel proteins) on, and samples (e.g., DNA, relevant protein products) of traditional and GMO reference materials (see press release, International Life Sciences Institute-ILSI: http://www.ilsi.org/). This should help ensure the quality and stability of reference standards and facilitate the standardization and distribution of materials and information needed for assay development.

Activities Of Trade Organization

GMO analytic procedures for foods and food ingredients were the subject of the recent "Workshop on Detection Methods for Novel Foods Derived from Genetically Modified Organisms" (Brussels, June 3–5, 1998), which was sponsored by the International Life Sciences Institute (ILSI) Europe in collaboration with the ILSI International Food Biotechnology Committee. The workshop involved government, industry, academic, and public sector scientists from 15 European countries and from North and South America and Asia.

At the request of Directorate General III, the EC administrative agency responsible for industry, information technology, and telecommunications, ILSI Europe will prepare an executive summary of the conclusions and recommendations made by the working groups and will publish the papers in Food Control.

The following proposals were made at the Workshop:

- A central repository, perhaps a European Union facility, should be created to house information (e.g., sequence data, primers, novel proteins) on, and samples (e.g., DNA, relevant protein products) of traditional and GMO reference materials. This should help ensure the quality and stability of reference standards and facilitate the standardization and distribution of materials and information needed for assay development.
- Globally harmonized sampling plans, perhaps through Codex Alimentarius, should be developed to facilitate sampling for GMO in raw foods and final food products.
- Although protein-based detection methods can be effectively used for detecting GMO in some foods and food products, they may be of limited value when GMO content is at or near likely test thresholds.
- Qualitative analysis of recombinant DNA is sufficiently sensitive and specific to establish the presence of recombinant DNA. However, quantitative methods will be necessary to establish whether the GMO content of a raw or final food product is or is not sufficient to trigger labeling as well as to certify that a food is GMO-free.
- Much uncertainty remains about establishing threshold detection levels that would trigger mandatory labeling of GMO content. The concept of genomic equivalence was proposed as a basis for establishing thresholds.
Conclusions

A summary of the status of the published official or validated methods and their significant differences are given in Table 2.

The published validated or official methods can be divided into "Product specific methods" and "Screening methods". Germany has published product-specific PCR methods, which are related to GM-potatoes, GM-Soybeans, GM-Lactobacillus curvatus and GM-Streptococcus thermophilus. The advantage of such methods is that the DNA extraction for each organism has been optimized and that specific target DNA is only detected in the respective GM-plants or organisms. The specificity of the reaction produces an amplicon that contains part of the regulatory gene sequence and part of the structural gene sequence, which is unique and does not occur in nature. These methods are very specific and are complementary to the screening method.

The screening methods validated and published in the European Union, Germany and Switzerland are based on the publication of Pietsch et al. [3], and the procedures set forth in each method differ only slightly. They have the advantage of being based on the search of the cauliflower mosaic virus 35S promoter and the Agrobacterium tumefaciens nos terminator, i.e. the most widely used promoter and terminator sequence in GM crops. Therefore, the screening primers are capable of detecting a large number of genetically modified crops. Moreover, the resulting amplicons are small enough to allow DNA amplification from degraded samples.

It must be stressed, however, that negative results do not exclude the presence of a GMO in the sample; they only exclude the presence of a 35S promoter and nos terminator. The diagram shown in Figure 1 illustrates the GMO analytical flow of the screening method (adapted from the Swiss food compendium 52B/4: Detection procedures for genetically modified Roundup Ready™ soybeans (RRS) in foods).

Table 2. Status of official/validated methods

<table>
<thead>
<tr>
<th>Published methods</th>
<th>EU</th>
<th>Switzerland</th>
<th>Germany</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 validated</td>
<td>1 official SLMB 52B</td>
<td>1 published by BgVV</td>
<td>4-official /§35 LMBG 02.02/97 (Streptococcus) 08.00/97 (Lactobacillus) 24.01/96-97 (Potato) 23.01.22/98 (Soybean)</td>
</tr>
<tr>
<td>Method type</td>
<td>Semi – generic PCR</td>
<td>Gene specific PCR</td>
<td></td>
</tr>
<tr>
<td>Target DNA Sequence</td>
<td>35S promoter and nos terminator</td>
<td>Potato: hygromycin gene Soya: 35S-RRS gene overlap</td>
<td></td>
</tr>
<tr>
<td>Confirmation Tests</td>
<td>Restriction enzyme analysis</td>
<td>Hybridization of amplicon with positive labeled DNA probe OR sequencing of the amplicon</td>
<td></td>
</tr>
<tr>
<td>QA Checks - DNA Yield</td>
<td>Agarose gel electrophoresis</td>
<td>A.G.E. or photometry</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>QA Checks – Amplifiability</td>
<td>PCR of lectin, zein or chloroplast gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methods in preparation</td>
<td>ELISA for RRS protein</td>
<td>Gene specific PCR for tomato</td>
<td></td>
</tr>
</tbody>
</table>
Summary

In the European Union and Switzerland, mandatory labeling of novel foods and food ingredients consisting of or containing GMOs is required according to Regulations EC 258/97 and 1139/98, and the Swiss Food Ordinance, respectively. Labeling requirements apply when it can be demonstrated that the food derived from the GMO has detectable differences from its conventional counterpart, i.e., the presence of newly introduced foreign DNA sequences, or newly expressed proteins. This has led most official and several industry laboratories to develop DNA analytical methods that can assist in the enforcement of the labeling provisions. Several questions remain open and need to be addressed on the standardization of official or validated methods and the availability of certified reference materials. The adoption of reference methods and material for DNA and proteins is essential for the development and validation of the method. The sensitivity and detection limits of each method are still being investigated and need to be defined. Threshold values that take into account the possibility of inadvertent mixing during agricultural production, shipping or processing have been established in Switzerland and have been proposed for the EU. Reliable quantitative PCR-based detection methods are being developed to address the introduction of GMO ceilings.
Figure 1. Analytical process for GMO analysis adapted from the Swiss Food Manual 52B/4.
References


10. § LMBG 23.01.22, March 1998: Detection of genetically engineered soy beans by amplification of the altered DNA sequence using the PCR (Polymerase Chain Reaction) and hybridization of the PCR product with a DNA probe.

11. § LMBG 24.01, January 1997 and 1996: Detection of genetically engineered potato by amplification of the altered DNA sequence using the PCR (Polymerase Chain Reaction) and hybridization of the PCR product with a DNA probe.

12. § LMBG 08.00, January 1997: Detection of genetically engineered Lactobacillus curvatus in raw sausage-meat by amplification of the altered DNA sequence using the PCR (Polymerase Chain Reaction) and hybridization of the PCR product with a DNA probe.

13. § LMBG 02.02, September 1997: Detection of genetic modification of Streptococcus thermophilus in yogurt by amplification of the modified DNA sequence by means of the Polymerase Chain Reaction (PCR) and hybridization of the PCR product with a DNA probe.


List of Internet Sites

Agency for Biosafety Research and Assessment of Technology Impacts of the Swiss Priority Program Biotechnology of the Swiss National Science Foundation.

http://www.bioweb.ch

Joint Research Centre (JRC), Environmental Institute, Consumer Protection and Food Unit, Ispra, Italy.

http://www.ei.jrc.it/
http://biotech.jrc.it/
http://food.ethz.ch/dmif-gen/

Institute for Reference Materials and Measurements (IRMM), Geel, Belgium

http://www.irmm.jrc.be/

Swiss Federal Office of Public Health (BAG)

http://www.admin.ch/bag/

International Life Sciences Institute (ILSI) Europe, International Food Biotechnology Committee

http://www.ilsi.org/index.htm

Reading Scientific Services Ltd. (RSSL) Reading, UK

http://www.rssl.co.uk/
APPENDIX I

INTERNET DATABASES ON GMO FIELD TRIALS

Given the frequent updates in the publicly available databases, a comprehensive list of internet addresses of public GMO field trials databases has been included:

Argentina
http://siiap.sagyp.mecon.ar/http-hsi/english/conabia/liuk.HTM

Australia

Brazil
http://www.fiocruz.br/cict/oquee/estrut/dect/bis/lib.htm

Canada
http://www.cfia-acia.agr.ca/english/ppc/biotech/field.html

Germany
http://www.rki.de/GENTEC/GENENG/GENTEC_E.HTM

EU
http://biotech.jrc.it/gmo.htm

Japan
http://ss.s.affrc.go.jp/docs/sentan/index.htm

USA
http://aphisweb.aphis.usda.gov/oa/new/ab.html

Worldwide
http://www.nbiap.vt.edu/
http://www.olis.oecd.org/biotrack.nsf/
http://www.gibip.org/database/index.html
http://binas.unido.or.at/binas/binas.html
http://www.nbiap.vt.edu/cfdocs/globalfieldtests.cfm
TOBACCO DNA EXTRACTION FOR PCR ANALYSIS

Introduction

This Section describes a practical approach for extraction of DNA from raw tobacco, or cigarette tobaccos for PCR analysis. It addresses the questions “How do varieties of tobacco (e.g. Bright, Orient, Burley etc.) differ from each other in respect to quality and quantity of DNA, that can be extracted, and how does curing and manufacturing of these tobaccos (e.g. air-cured, sun-cured, expanded, reconstituted tobaccos) influence DNA quantity and quality?”

Methods

DNA Extraction and Purification

Initially, a modified CTAB-Method was used for DNA-Extraction.

Depending on the quality of DNA in respect to the PCR the following sequence of additional methods for purification of the DNA were used on the same sample:

1. Wizard® DNA Clean-Up System (Promega)
2. MicroSpin™ S-300HR column (Amersham Pharmacia Biotech)
3. Wizard® DNA Clean-Up System
4. MicroSpin™ S-300HR column

After each step the DNA was again tested for quantity and quality in respect to PCR.
DNA Quantity Test

For testing the quantity of DNA, an aliquot of the extracted sample was separated on a 1% Agarose gel stained with Ethidium bromide. Quantity was calculated by comparison of fluorescence of the sample to calf-thymus DNA standards also loaded onto the same gel in a different slot.

DNA Quality Test

Quality of DNA was checked mainly by its capability to inhibit PCR. The samples were spiked with about 20 copies of 35S-DNA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>35S-af1</th>
<th>CAC CTA CAA ATG CCA TCA TTG C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35S-ar1</td>
<td>GGG TCT TGC GAA GGA TAG TG</td>
</tr>
</tbody>
</table>

50 cycles:  
94°C  25sec  
62°C  30sec  
72°C  45sec

Fragment: 207 bp

Results

Usually each purification step resulted in loss of DNA, and especially fragments smaller than 175bp were lost. As PCR screening methods for GMOs usually amplify fragments of about 200bp in size, the loss of small fragments should not interfere with e.g. 35S-screening.

Results are summarised in Table 1.

An additional observation is that contamination of raw material with fungi can result in very strong inhibition of PCR.
Table 1. Summary of results.

<table>
<thead>
<tr>
<th>Type of curing / manufacture</th>
<th>DNA after CTAB (ng per g sample)</th>
<th>DNA after last purification step (ng per g sample)</th>
<th>PCR</th>
<th>Purification Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bright Flue-cured</td>
<td>1000-3000 &lt;2000</td>
<td>Yes</td>
<td></td>
<td>Wizard® 1 MicroSpin™ 2 Wizard® 3 MicroSpin™ 4</td>
</tr>
<tr>
<td>Dark-fired Fire-cured</td>
<td>&lt;500 n.d.</td>
<td>No</td>
<td></td>
<td>100% 100% 100% 100%</td>
</tr>
<tr>
<td>Burley Air-cured</td>
<td>500-1500 &lt;1500</td>
<td>Yes</td>
<td></td>
<td>100% 50% 100% 100%</td>
</tr>
<tr>
<td>Orient Sun-cured</td>
<td>500-1500 &lt;1000</td>
<td>Yes</td>
<td></td>
<td>100% 100% 100% 100%</td>
</tr>
<tr>
<td>Stems</td>
<td>1000 &lt;1000</td>
<td>Yes</td>
<td></td>
<td>100% 100% 100% 100%</td>
</tr>
<tr>
<td>Reconstituted</td>
<td>&lt;1000 &lt;500</td>
<td>Yes</td>
<td></td>
<td>100% 100% 100% 100%</td>
</tr>
<tr>
<td>20-40% Burley 60-80% Bright</td>
<td>CO₂ expanded 500-1000 &lt;1000</td>
<td>Yes</td>
<td></td>
<td>100% 20%</td>
</tr>
<tr>
<td>Cigarettes</td>
<td>500-3000 &lt;3000</td>
<td>Yes</td>
<td></td>
<td>100% 100% 5%</td>
</tr>
<tr>
<td>Cigars</td>
<td>1500-3000 &lt;2500</td>
<td>Yes</td>
<td></td>
<td>100% 100% 20%</td>
</tr>
</tbody>
</table>

a) DNA was usually extracted from 2g material.
b) 100% of all templates had to be purified at least once with Wizard system not to inhibit PCR.
c) 20% of all templates had to be purified at least once with Wizard and once with Microspin not to inhibit PCR.
d) 100% of all templates had to be purified at least twice with Wizard and once with Microspin not to inhibit PCR.
e) 100% of all templates had to be purified at least twice with Wizard and twice with Microspin. In the case of dark fired tobacco PCR was still inhibited.
Summary

• In general, too much DNA-matrix inhibits PCR. However, in the case of tobacco it is not yet clear, whether this depends only on the amount of DNA-matrix used or also on some compounds from tobacco, which could not be separated from DNA during extraction and purification and which inhibit PCR.

• The more tobacco is processed, either by curing or by manufacturing, the less DNA can be extracted.

• Quality of extracted DNA depends firstly on the variety of tobacco and secondly on the manufacturing process.

• Dark-fired tobaccos could not be analyzed by PCR; even when only very low amounts of samples were applied, PCR was inhibited.
APPENDIX III

CHINESE METHOD FOR SCREENING GENETICALLY MODIFIED TOBACCO

Purpose................................................................................................................................... 93
Introduction................................................................................................................................ 94
  Kanamycin Resistance and nptII PCR Testing............................................................... 94
  35S and nos PCR Testing ............................................................................................. 94
  Confirmation Tests ...................................................................................................... 95
  Detection of Specific Transgenes................................................................................... 95
General Requirements............................................................................................................ 96
  Duplication.................................................................................................................. 96
  Control Samples........................................................................................................... 96
  Accuracy Check............................................................................................................ 97
  Contamination............................................................................................................. 97
  Safety Precautions....................................................................................................... 97
Screening by Kanamycin Resistance..................................................................................... 97
PCR Testing for nptII, 35S and nos ....................................................................................... 99
  Sample Preparation ..................................................................................................... 99
  DNA Extraction ........................................................................................................... 99
  Determination of DNA Yield and Purity................................................................. 102
  PCR Process ............................................................................................................... 103
  Analysis of the PCR Products.................................................................................... 105
Confirmation........................................................................................................................ 106
  Restriction Analysis of 35S and nos Amplified Fragments......................................... 106
  Nested or Semi-nested PCR....................................................................................... 108
Determination of Specific Transgenes................................................................................. 110
  Tobacco Mosaic Virus Coat Protein........................................................................... 110
  Cucumber Mosaic Virus Coat Protein....................................................................... 110
  Potato Virus Y Coat Protein ..................................................................................... 110
  TMV 54kD Gene ....................................................................................................... 111
  Bt Gene ....................................................................................................................... 112
References............................................................................................................................ 113
PURPOSE

Worldwide agreement on the use of genetically modified (GM) plants including tobacco has not yet been achieved. In order to confirm that the product from tobacco fields in China, especially for export, is GM-free, we carry out screening for GM tobacco.

The methods described here aim to detect genetic modification in tobacco material, such as seeds, seedlings, fresh leaf and cured tobacco leaf. They provide a means of identifying a limited number of genes that have been reported to be inserted in certain tobacco varieties.

The methods have been developed in our laboratory based on published work and many detection methods for GM tobacco from other laboratories, especially the screening methods for cured tobacco leaf. These have provided acceptable results in our laboratory with positive control samples of genetically modified (GM) tobacco.

Introduction

Three genes (nptII, 35S, nos) are commonly used as markers in plant transformation research. These are used for early screening of transgenic plants.

Kanamycin Resistance and nptII PCR testing

As a selection gene, neomycin phosphotransferase (which encodes resistance to kanamycin) is popularly used in genetically modified tobacco. According to the results of experiments in our laboratory, cells of GM tobacco can tolerate more than 100 ppm kanamycin, but the normal tobacco cell will became yellow and die after more than 7 days in this condition. Thus kanamycin resistance can be used to screen GM tobacco seeds.

Since the nptII gene was transfered to the tobacco genomic DNA from a bacterium, it cannot be found in the non-GM plant DNA. Using the nucleotide sequence of nptII, primers were designed to test extracted DNA of tobacco seed samples. This is not used to test cured tobacco leaf since there are bacteria which may contain the nptII gene on the surface of the cured leaf which can result in false positive results.

35S and nos PCR Testing

As 35S and nos are the most frequently used plant promoter and terminator, they can be used in a PCR test. This is capable of detecting modification in a majority of currently-available GM plant materials. Also the screening result cannot be affected by bacteria on the fresh or cured leaf.
Confirmation Tests

After PCR screening nptII, 35S or nos, some samples may give a positive result. Confirmation tests aim to avoid the risk of false positives. The tests, which include restriction analysis and nested- or semi-nested PCR, seek to confirm the presence of the 35S and nos sequences in these samples, especially in cases where the initial sample result appears less intense than the positive control.

Detection of Specific Transgenes

Reported work on GM tobacco has introduced resistance genes to virus or insects, including the following:

- Tobacco Mosaic Virus coat protein gene (TMV-CP),
- Cucumber Mosaic Virus coat protein gene (CMV-CP),
- Potato Virus Y coat protein gene (PVY CP),
- Bt insect resistance crystal protein gene.
GENERAL REQUIREMENTS

Duplication

In order to obtain a reliable result, each sample is analysed in duplicate, i.e. extraction is performed on two separate test samples and two aliquots of extract are submitted to PCR analysis.

Control Samples

Positive Control
The positive control samples are previously-tested GM tobacco samples obtained from the Institute of Microbiology Academia Sinica and other institutes, and have been extensively tested in our laboratory according to the present procedure.

Negative Control
The negative control samples were previously-tested non-GM tobacco (Seed origin from Henan Agriculture Academic Tobacco Institute).

Reagent Blank
No tobacco material is included in the sample. It is prepared using the same reagents and analytical procedures as for the test samples to control the quality of the extraction buffer.

Extraction Blanks
During the grinding of the samples, two microtubes are positioned next to the work place and subsequently submitted to PCR in order to control sample cross-contamination.
Accuracy Check

After every PCR procedure, the results are accepted only if a single band in the right position relative to the DNA size marker is obtained from the positive control, and at the same time there are no bands from the negative controls, reagent blank, or extraction blanks. Otherwise, the procedure is repeated. When samples yield weak bands on gel-electrophoresis (not as bright as the positive control), or more than one band is found on the gel, the DNA extraction and PCR should be repeated.

Contamination

One of the major problems in PCR-based methods is the accidental contamination of samples and reagents with DNA from previous experiments or from non-target sources especially from the positive control. Even minute cross-contamination is very likely to lead to false-positive results. To minimize such risks, sample preparation, DNA extraction, PCR and gel electrophoresis are carried out in different laboratories and the working surfaces are decontaminated with UV light (254 nm) overnight.

All samples are handled using sterile DNA- and DNAase-free equipment, wearing gloves at all times and changing the tips of the micro-pipette after each operation.

Airborne dust and aerosols may be formed during grinding or handling of tobacco samples, so laboratories should be well ventilated and samples are handled under a chemical fume hood area provided with strong exhaust ventilation.

Safety Precautions

A laboratory coat, safety glasses and gloves are worn throughout all steps of the procedure. Special care and chemical-resistant gloves, safety glasses and a mask must be used when working with ethidium bromide (EB), which should be used only in the chemical fume hood.

Screening by Kanamycin Resistance

Method 1.
1. Put seeds of each sample in a 1.5 ml micro tube.
2. Add 1.0 ml of 70% ethanol, then pipette out immediately.
3. Add 1.0 ml of 20% Clorox and soak for 30 min. Invert vials regularly.
4. Wash 4 times with sterile distilled water.
5. Distribute seeds evenly on plates containing ½ MS medium using a 1 ml pipette with a tip widened by cutting.
6. Transfer the germinating seeds to ½ MS medium plates containing kanamycin at 0, 50, and 100 ppm. Observe growth on every treatment after 14 days.
Method 2.

Steps (1)–(4) are as Method 1.
5. Steam sterilise some 9cm filter paper, and filter sterilise the kanamycin solution.
6. Place a double layer of filter paper in a petridish and add 4 ml sterile water with 0, 50, 100 ppm kanamycin solution. Distribute the sterilized seeds evenly and incubate at 26.5°C in continuous light. Observe growth after 10 days.
Screening PCR for \textit{nptII}, 35S and \textit{nos} genes

Sample preparation

Seeds and Fresh Leaf
Sterilise seeds as described above. Cultivate (without adding kanamycin) for more than 5 days in the incubator.

Cured Leaf
See the protocol for DNA isolation from tobacco cured leaf (Below).

DNA extraction

Materials and Reagents

High speed freezer centrifuge
Micropipettes and tips
Gel electrophoresis system and Transilluminator
Analytical balance accurate to 0.1 mg
Heating bath (65°C)
Spectrophotometer
Microwave oven and heating plate
Steam sterilizer
Ultra-purity water system
Eppendorf tubes
CTAB
Ethanol
Agarose
10xTBE buffer
EB (Ethidium bromide)
Sample loading buffer
Dneasy plant mini kit (Qiagen)
RNase
DNA ladder
DNA Isolation from Seeds and Fresh Leaf

1. Germinate seeds on filter paper. Then grind 200 mg seedlings into powder in liquid nitrogen in a mortar and pestle (pre-chilled at –85°C).
2. Transfer the powder to a microfuge tube, and add 2x CTAB extraction buffer (2% CTAB (w/v); 100mM Tris (pH8.0); 20 mM EDTA (pH8.0); 1.4M NaCl; 1% PVP; pre-warmed to 65°C) according to the proportion of 1µl/mg. Heat the mixture in a 65°C water bath for 1-3 mins.
3. Add an equal volume of chloroform /isoamyl alcohol (24:1, v/v). Mix gently but thoroughly and centrifuge at 11000g for 4 mins.
4. Transfer the top phase to a new microtube and add 1/10 volume 10% CTAB(w/v); 0.7 M NaCl (pre-warmed to 65°). Add a second equal volume of chloroform /isoamyl alcohol (24:1, v/v) and centrifuge.
5. Transfer the top phase to a new microtube and add one volume of CTAB precipitation buffer (1% CTAB; 50mM Tris (pH 8.0); 10mM EDTA (pH8.0)) followed by gentle mixing. Centrifuge for 8min at 15000g.
6. Rehydrate the pellet in high salt TE buffer (10mM Tris (pH8.0); 1mM EDTA (pH8.0); 1M NaCl), in a 65°C water bath for 10 min.
7. Reprecipitate the nucleic acid with two volumes of ethanol, and 8 min of centrifugation .
8. Wash the pellet with 80% ethanol and dry in a desiccator.
9. Rehydrate the nucleic acid pellet in 50µl 0.1xTE buffer (1mM Tris (pH8.0); 0.1mM EDTA).

DNA Isolation from Cured Leaf

(A) Modified CTAB Method

1. Add 700µl 1xCTAB extraction buffer, mix and heat for 10 min at 65°C.
2. Add 700µl chloroform/isoamyl alcohol and centrifuge at 12000g for 4 min.
3. Take the top phase and add 1/10 volume of 10% CTAB extraction buffer. Re-extract with chloroform/isoamyl alcohol and centrifuge.
4. Repeat Step 3. Then add one volume of CTAB precipitation buffer and centrifuge at 15000g for 8 min.
5. Rehydrate the pellet in high salt TE buffer at 65°C for 5-10 min.
6. Add two volumes of ethanol and hold at 70°C for 30 min.
7. Centrifuge for 8 min. at 15000g and rehydrate the pellet in 70µl TE buffer.

(B) Modified Protocol for DNA Isolation from Plant Tissue with the DNEASY Plant Mini Kit (Quiagen Inc.)

1. Place half of the sample (max. 20g) in a plastic bag, immerse in liquid nitrogen and grind with a cell disrupter (Bioreba) to obtain a homogenous powder. Transfer to a labeled 50ml sterile centrifuge tube. Weigh out 20-25mg of ground dry tobacco power into a 1.5ml microtube.
2. Prepare the following Stock solution: 400µl AP1 + 5µl Rnase (100µg/µl) per sample to be extracted. Add 400µl Stock solution to the 20-25mg tobacco powder. Vortex to obtain a homogenous mixture.
3. Incubate the mixture 10 minutes at 65°C.
4. Add 130µl AP2 to the lysate, and incubate for 5 minutes on ice.
5. Remove from ice and pour the lysate onto the QIAshedder spin column, sitting on a 2ml collection tube. Centrifuge for 2 minutes at 14,000rpm.
6. Transfer the flow-through fraction to a new tube without disturbing the pellet.
7. Add 225µl AP3 buffer and 450µl ethanol (room temperature) to the 450µl lysate.
8. Apply half of the solution obtained in Step 7 onto the Dneasy mini spin column sitting in a 2ml collection tube. Centrifuge for 2 minutes at 10000rpm.
9. Repeat Step 8 with the remaining solution from Step 7.
10. Place Dneasy column in a new 2ml collection tube and add 500µl AW buffer onto the Dneasy column and centrifuge for 2 minutes at 10000rpm.
11. Add 500µl AW buffer onto the Dneasy column and centrifuge for 2 minutes at 14 000rpm.
12 Transfer the Dneasy column to a new 1.5ml microtube and add 130µl preheated (65°C) AE buffer onto the center of the Dneasy column and centrifuge for 2 minutes at 10000rpm.
Determination of DNA Yield and Purity

Electrophoresis Observation

Nucleic acid extracted from each sample is subjected to electrophoresis on an 0.8% agarose gel. DNA concentration and length is estimated by comparison of the position and brightness of the bands on the gel with a dilution series of lambda DNA (0, 10, 25, 50, 100, 200 ng/µl).

Measurement by UV spectrophotometer

The yield and purity of the nucleic acid can be estimated by the measurement of OD at 260nm and 280nm using a UV-spectrophotometer.

1. Transfer 5µl of the DNA extraction sample into a microtube. Add deionized water to 1ml and mix completely. Then fill into the cup of the spectrophotometer.
2. Correct the zero of the spectrophotometer with distilled water.
3. The Optical Density is measured at 260nm and 280nm alternately. Calculate the DNA concentration by OD_{260nm} x dilution fold x 50/1000 (µg/µl).
4. If the value of OD_{260}/OD_{280} is more than 1.8, this indicates the presence of RNA in the extract. If the value is less than 1.6 this indicates protein or phenol in the DNA extraction sample.

Testing Tobacco Chloroplast Non-coding DNA

The quality of DNA extraction can also be checked by detection of a tobacco chloroplast non-coding DNA region sequence using PCR amplification methods.

Chloroplast Universal Primer:

<table>
<thead>
<tr>
<th>Plant</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>5' CGA AAT CGG TAG ACG CTA CG -3'</td>
</tr>
<tr>
<td>-2</td>
<td>5' GGG GAT AGA GGG ACT TGA AC -3'</td>
</tr>
</tbody>
</table>

PCR of tobacco chloroplast DNA with these primers yields a product of 550 bp, which can be used as a quality marker for DNA preparation. As the PCR assay is very sensitive, this method will determine if the DNA extract can be used as a template for the GM screening assays. This is particularly useful following the DNA extraction of cured tobacco leaf sample.
PCR Process

Material and Reagents
Thermocyclers: Thermolyyn Gene Amp PCR 9600
Micropipette (brand and Reinin) and tips
Sterile deionized water (Millipore, Ultrafiltered)
Taq enzyme; 10xbuffer; Mg$^{2+}$; dNTP (Promega)
Synthesized Oligonucleotide Primers (COL purity, storage in -40°C freezer)

Primers for Screening
For GM tobacco screening the following DNA sequences or genes are tested:

- CaMV 35S-promoter sequence
- A. tumefaciens nos-terminator sequence
- nptII sequence

Primers were custom synthesized and HPLC-purified by the commercial supplier, and dissolved in water to give a 400μM stock solution.

A: 35S-promoter

A 195bp PCR product is used as a marker to detect the presence of the promoter sequence from Cauliflower Mosaic Virus

35S-1  5’ GCT CCT ACA AAT GCC ATC A -3
35S-2  5’ GAT AGT GGG ATT GTG CGT CA -3
B: nos-terminator

A 180bp PCR product is used as a marker to detect the presence of the nos-terminator sequence from Agrobacterium tumefaciens.

NOS -1 5’- GAA TCC TGT TGC CGG TCT TG -3
NOS -2 5’- TTA TCC TAG TTT GCG CGC TA -3

C: nptII, Neomycin phosphotransferase II

A 411bp PCR product can be used as a marker to detect the nptII gene sequence using the following primers.

NPTII-1 5’ GCC CTG AAT GAA CTG CAG GAC GAG GC -3’
NPTII-2 5’ GCA GGC ATC GCC ATG GGT CAC GAC GA -3’

A 785bp PCR product can be used as a marker to detect the nptII gene sequence using the following primers.

NPTII-3 5’ GAA CAA GAT GGA TTG CAC GC -3’
NPTII-4 5’ GAA GAA CTC GTC AAG AAG GC -3’

A 699bp PCR product can be used as a marker to detect the nptII gene sequence using the following primers.

NPTII-3 5’- AGA GGC TAT TCG GCT ATG ACT GGG C -3’
NPTII-4 5’- CGG GAG CGG CGA TAC CGT AAA GC -3’
PCR Reaction

The reaction is performed in a 0.25ml microtube. The following components are added sequentially to a pre-chilled sterile microfuge tube and the tube was finger-vortexed after each addition.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Loading Order</th>
<th>Volume µl</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10xBuffer</td>
<td>1</td>
<td>5.0</td>
<td>1xBuffer</td>
</tr>
<tr>
<td>dNTP</td>
<td>2</td>
<td>1.5</td>
<td>200µM</td>
</tr>
<tr>
<td>Primer 1</td>
<td>3</td>
<td>2.5</td>
<td>25pmol/rxn</td>
</tr>
<tr>
<td>Primer 2</td>
<td>4</td>
<td>2.5</td>
<td>25pmol/rxn</td>
</tr>
<tr>
<td>DNA Template</td>
<td>5</td>
<td>1.0</td>
<td>10ng/rxn</td>
</tr>
<tr>
<td>Water</td>
<td>6</td>
<td>18.5</td>
<td></td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>7</td>
<td>3</td>
<td>2.5-3u/rxn</td>
</tr>
<tr>
<td>Mineral Oil</td>
<td>8</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

After reagents 1-6 are added the reaction solution is mixed thoroughly and centrifuged at 6500rpm for 30 sec. The tube is placed into the Air thermocycler to perform the pre-denaturation at 94°C for 7 min. Then Taq DNA Polymerase and mineral oil are added. The capillary tube is centrifuged at 6500rpm for 30 sec. Finally the loaded tube is placed into the reaction chamber of the Air thermocycler and the program is run. After the run the PCR tube is stored in a –40°C freezer.

PCR Conditions

For the 35S test, the following cycle program is used:
- Pre-cycle: Hold temperature at 95°C for 7 minutes,
- Cycle: Cycle 35 times as follows
  - Denaturation at 94°C for 30 sec.,
  - Annealing at 62°C for 45 sec.,
  - Extension at 72°C for 90 sec.,
- Post-cycle: Hold at 72°C for 7 minutes.

In all PCR reactions a positive (DNA extracted from GM tobacco), a negative (DNA extracted from non-GM tobacco), a reagent blank (no DNA), and an extraction blank are tested with the same primer set.

Analysis of the PCR Products

Material and Reagents

Gel electrophoresis system
Microwave oven
Transilluminator (UV light, 312nm)
Micropipette and tips
5xTBE buffer
DNA ladder (100bp) length standard
Sample loading buffer (1.5g Ficoll type 400, 25mg Bromophenol Blue in 10ml water).

**Gel Electrophoresis and Documentation**

Agarose (1.5% for amplicons of 250-600bp) is completely dissolved in boiling 0.5xTBE buffer. After cooling to about 45°C, the agarose solution is poured into the gel mould, fitted with a comb. The gel is cooled and the comb removed. The gel is placed in the electrophoresis chamber and covered with about 5mm of TBE buffer. 2µl of loading buffer is added to 10µl of the amplified PCR solution, and the mixture is loaded onto the gel in 0.5xTBE buffer. On each gel plate at least 3 lanes must be left at each side of the plate for positive control, negative control, and DNA ladder.

After loading, the gel is run for 2 hours at 100V in 0.5xTBE buffer. Finally the gel is stained in 0.5µg/ml ethidium bromide for about 40 minutes and the DNA bands visualised using a UV transilluminator.

**Confirmation test**

If a sample is PCR-positive either the promoter or the terminator sequence, a restriction enzyme and sequence analysis of the amplified DNA fragment can be performed to confirm the results. To increase the sensitivity of the screening, nested- or semi-nested PCR can be applied. When the PCR product bands of the samples are less intense than the positive control, the confirmation test must be done.

**Restriction Analysis of 35S and nos Amplified Fragments**

**Material and Reagents**

*Asp700 (XmnI)* restriction enzyme
*NsiI* restriction enzyme
Gel electrophoresis system
Microwave oven
Transilluminator (UV light, 312nm) and documentation device
Micro-pipette and tips
Agarose
5xTBE buffer
Sample loading buffer
DNA ladder
Ethidium bromide
Restriction Enzyme Reaction Preparation

Besides the produced DNA fragment, PCR reactions contain reagents such as Taq DNA Polymerase, dNTP, DNA template and other materials which can inhibit the activity of the restriction enzyme. These can be removed by DNA purification as follows;

Method 1
Remove the top phase (mineral oil) with a micro-pipette. Add 2 volumes of chloroform and vortex. Centrifuge for 5 min. at 12000g, and collect the top phase to a new tube.

Method 2.
Transfer the PCR product to another sterile microtube, and add 100µl ethanol to precipitate the DNA fragments. The pellet is rehydrated in 50µl high salt TE buffer.

Method 3.
Purify the amplified DNA fragment using a commercial kit (Promega).
Restriction Enzyme Reaction

1. Transfer 10μl of the purified PCR product to a new tube. Add 10x restriction enzyme buffer and 1μl restriction enzyme (4-10units). Vortex and incubate at 37°C for 3 hours.
2. Mix 2μl restriction enzyme cut product with 1μl loading buffer and examine by 1.5% agarose gel electrophoresis.

35S Promoter

If the sample is 35S promoter positive, an Asp700 (XmnI) restriction enzyme analysis of the amplified DNA fragment is performed. The 195bp amplified fragment can be cut into two pieces of 115bp and 80bp length with the restriction enzyme Asp700. This reaction can be performed by adding 5units of Asp700 enzyme to 10μl PCR reaction solution and incubating the solution for 3 hours at 37°C. After that, the products are analyzed by electrophoresis through a 1.5 % agarose gel in 0.5xTBE buffer followed by staining in ethidium bromide and visualization of the DNA bands using a UV transilluminator.

nos terminator

If the sample is nos-terminator positive, a NsiI restriction enzyme analysis of the amplified DNA fragment is performed. The 180 bp amplified fragment can be cut into two pieces of 96bp and 84bp length with the restriction enzyme NsiI. This can be performed by adding 5 units of NsiI enzyme to 10μl PCR reaction solution and incubating the solution for 2 hours at 37°C. The products are analysed by electrophoresis through a 2.5% agarose gel in 0.5xTBE buffer followed by staining in ethidium bromide and visualization of the DNA bands using a UV transilluminator.

Nested or Semi-nested PCR

If the amplified PCR fragment is very weak or not visible (the presence of the transgene is uncertain), it is recommended to perform a nested or semi-nested PCR for detection of 35S or nos- sequences.

There are two pairs of primers in the nested PCR assays, one being the same as for the normal PCR, and is added into the reaction solution to perform the first PCR. Another pair of primers called specific primers is added into the the second PCR reaction. The sequence of the specific primers is designed according to the sequence of the target DNA such that they fall within the first amplified fragment. Thus the fragment can be used as the template of the second PCR process. It is necessary that the fragment amplified by the second primers is shorter than that of the first one. PCR products are analysed by electrophoresis through a 1.5% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator. By using nested PCR, the sensitivity of PCR screening is increased.
Nested PCR Assay of 35S

Primer:

- 35S-nest-1: 5' CGT TCA CCT AAC TAC ACT AT 3'
- 35S-nest-2: 5' GTG ACT GCC GTC TCC GTA GA 3'

The product of the 35S nested PCR is 110 bp in length.

Nested PCR Assay of nos-terminator

The product of nos nested PCR is 100 bp in length.

Nested PCR Assay of nptII

The products obtained are very different when different primers are used in the initial PCR process. In the screening methods of tobacco cured leaf described above, primers NPTII-1 and NPTII-2 are usually used. The second pair of primers (Below) produce a product of the nested PCR of 209bp.

- NPTII-nest-1: 5' CTG GCT GCT ATT GGG CGA AG 3'
- NPTII-nest-2: 5' TCG TCC AGA TCA TCC TGA TC 3'
Determinations of specific transgenes

**TMV - CP: Tobacco Mosaic Virus Coat Protein**

A 327bp PCR product is used as a marker to detect the presence of the TMV-cp gene sequence with the following primers:

- **TMV1**: 5' - GTG TTC TTG TCA TCA GCG TGG GC -3'
- **TMV2**: 5' - CAC CGT TGC GTC GTC TAC TCT AC G -3'

The following cycle program is used:
- **Pre-cycle**: Hold temperature at 95°C for 5 minutes,
- **Cycle**: Cycle 30 times as follows
  - Denaturation at 94°C for 1 minute,
  - Annealing at 55°C for 1 minute,
  - Extension at 72°C for 2 minutes,
- **Post-cycle**: Hold at 72°C for 7 minutes.

**CMV – CP: Cucumber Mosaic Virus Coat Protein**

A 202 bp PCR product is used as a marker to detect the presence of the CMV-cp gene sequence with the following primers:

- **CMV1**: 5' - AAG ACG TTG GCA GCT GGT CG -3'
- **CMV2**: 5' - CTC GAA TTT GAA TGC GCG AA -3'

A 263 bp PCR product can be used as a marker to detect the presence of the CMV cp gene sequences with primers CMV3 and CMV4.

- **CMV3**: 5' - ACC CAA CCT TTG TAG GGA GTG AGC G -3'
- **CMV4**: 5' - ACA TAG CAG AGA TGG CGG CAA CG -3'

The PCR cycle used is as for TMVcp, with the exception that the Pre-cycle phase is:
- **Pre-cycle**: Hold temperature at 94°C for 2 minutes

**PVY CP: Potato Virus Y Coat Protein**

A 362 bp PCR product can be used as a marker to detect the presence of the PVY-cp gene sequence using the primers PVY1 and PVY2:

- **PVY1**: 5' - GAT ATT TCA AAT ACT CGG GCA -3'
- **PVY2**: 5' - GCA TAA CGC GCT AAA CCC AC -3'
A 272 bp PCR product can be used as a marker to detect the presence of the PVYcp gene sequence using the primers PVY3 and PVY4:

\[
\begin{align*}
\text{PVY3} & : 5' - \text{CTC AGA TGT TGC AGA AGC GT} -3' \\
\text{PVY4} & : 5' - \text{CCT CGG TGG TGT GCC TCT CT} -3'
\end{align*}
\]

The PCR cycle program used is the same as for the CMVcp gene.

**TMV 54 kD Gene**

A 295 bp PCR product can be used as a marker to detect the presence of the TMV 54 kD gene sequence with following primers:

\[
\begin{align*}
\text{TMV54 1} & : 5' - \text{GAG TTG TCT GGC ATC ATT GA} -3' \\
\text{TMV54 2} & : 5' - \text{ACA ATG GTC AAA GCC GGG TA} -3'
\end{align*}
\]

The following cycle program is used:

- **Pre-cycle:** Hold temperature at 95°C for 5 minutes,
- **Cycle:** Cycle 35 times as follows
  - Denaturation at 94°C for 1 minute,
  - Annealing at 60°C for 1 minute,
  - Extension at 72°C for 2 minutes,
- **Post-cycle:** Hold at 72°C for 7 minutes.
**Bt gene**

A 299bp PCR product can be used as a marker to detect the presence of the Bt gene sequence using the following primers:

- **BT -1** 5' - CCC ACT AGT TAA CAA TTT GAT TGG A -3'
- **BT -2** 5' - CCG GAA GCT TTA GGA CTG TAG GT -3'

The following cycle program is used:
- Pre-cycle: Hold temperature at 94°C for 5 minutes,
- Cycle: Cycle 30 times as follows
  - Denaturation at 94°C for 45 sec.,
  - Annealing at 55°C for 1 minute,
  - Extension at 72°C for 2 minutes,
- Post-cycle: Hold at 72°C for 7 minutes.
REFERENCES


## GLOSSARY OF TERMS

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S</td>
<td>Promoter from Cauliflower Mosaic Virus, commonly used in transgene constructs</td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism, a method of DNA fingerprinting</td>
</tr>
<tr>
<td>agarose gel</td>
<td>A medium for DNA electrophoresis</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>Bacterium used as a vector for plant transformation</td>
</tr>
<tr>
<td>amplicon</td>
<td>DNA fragment produced by PCR amplification of a specific target region</td>
</tr>
<tr>
<td>amplification</td>
<td>Increasing the concentration of a specific DNA fragment to detectable levels by PCR</td>
</tr>
<tr>
<td>annealing, anneal</td>
<td>Interaction between two DNA molecules to form a double strand (see also hybridisation)</td>
</tr>
<tr>
<td>base pair (bp)</td>
<td>Paired nucleotides in a DNA molecule. Used as a unit of length of a DNA strand.</td>
</tr>
<tr>
<td>Bt</td>
<td>Bacillus thuringensis, or the insecticide produced by this bacterium</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>construct</td>
<td>Spliced DNA molecule prepared for transformation</td>
</tr>
<tr>
<td>cry1A</td>
<td>One gene encoding a Bt insecticidal protein</td>
</tr>
<tr>
<td>cycle</td>
<td>The repetitive step of a PCR reaction</td>
</tr>
<tr>
<td>denaturation (of DNA)</td>
<td>Separation of double-stranded DNA into single strands. Necessary for replication and PCR amplification</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>DNA, Deoxyribonucleic acid</td>
<td>Linear molecule composed of two strands of nucleotides. Carrier of genetic information.</td>
</tr>
<tr>
<td>electrophoresis</td>
<td>Process used to separate and identify DNA fragments, by applying an electric field.</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay. Protein detection method</td>
</tr>
<tr>
<td>endogenous</td>
<td>Occurring naturally within the organism</td>
</tr>
<tr>
<td>EPSPS, EPSP</td>
<td>Enol puruvyl shikimate-3-phosphate synthase. Enzyme inhibited by glyphosate</td>
</tr>
<tr>
<td>fragment</td>
<td>A DNA molecule of defined length</td>
</tr>
<tr>
<td>gene</td>
<td>Unit of genetic information, comprising DNA and usually encoding a protein</td>
</tr>
<tr>
<td>genetic engineering, genetic modification</td>
<td>Producing a novel organism with desirable characteristics by transformation using DNA constructs in the laboratory.</td>
</tr>
<tr>
<td>glyphosate</td>
<td>Herbicide marketed as RoundUp.</td>
</tr>
<tr>
<td>GM</td>
<td>Genetically modified</td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically modified organism</td>
</tr>
<tr>
<td>homologous / homology</td>
<td>DNA molecules having identical or highly similar sequence</td>
</tr>
<tr>
<td>hybridisation</td>
<td>Annealing of homologous DNA molecules</td>
</tr>
<tr>
<td>junction sequence</td>
<td>Region between two spliced sections of a transgene</td>
</tr>
<tr>
<td>multiplex PCR</td>
<td>PCR of multiple target genes in a single reaction tube.</td>
</tr>
<tr>
<td>neomycin phosphotransferase</td>
<td>Gene conferring Kanamycin resistance</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>nested PCR</td>
<td>Sequential PCR focussing on a specific <strong>target DNA</strong> region</td>
</tr>
<tr>
<td>nopaline synthase</td>
<td>Enzyme from <em>Agrobacterium tumefaciens</em></td>
</tr>
<tr>
<td>nos</td>
<td><strong>Nopaline synthase gene</strong> used as a source of promoter or terminator sequences</td>
</tr>
<tr>
<td>NPTII, nptII, neo</td>
<td><strong>Neomycin phosphotransferase gene</strong></td>
</tr>
<tr>
<td>nucleotide</td>
<td>Basic repeat unit of a <strong>DNA</strong> molecule</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction. Method of detecting <strong>DNA sequences</strong>, comprising multiple cycles of amplification</td>
</tr>
<tr>
<td>primer</td>
<td>Short single-stranded <strong>DNA</strong> molecule used in <strong>PCR</strong> to specify the <strong>target sequence</strong></td>
</tr>
<tr>
<td>probe</td>
<td>A labelled <strong>DNA</strong> molecule used in <strong>hybridisation</strong> assays.</td>
</tr>
<tr>
<td>promoter</td>
<td><strong>DNA sequence</strong> regulating <strong>gene</strong> expression</td>
</tr>
<tr>
<td>restriction analysis</td>
<td><strong>DNA</strong> analysis by enzymatic cleavage at <strong>specific sequences</strong></td>
</tr>
<tr>
<td>selectable marker</td>
<td><strong>Gene</strong> allowing selection of <strong>GM</strong> from non-GM plants (e.g. by conferring antibiotic resistance)</td>
</tr>
<tr>
<td>sequence</td>
<td><strong>DNA</strong> molecule whose identity can be described by the order of <strong>nucleotides</strong> along it</td>
</tr>
<tr>
<td>sequencing</td>
<td>Determining the order of <strong>nucleotides</strong> along a <strong>DNA</strong> strand.</td>
</tr>
<tr>
<td>Southern blotting</td>
<td>Transfer of <strong>DNA</strong> onto a membrane for a <strong>hybridisation</strong> assay.</td>
</tr>
<tr>
<td>spliced</td>
<td>Conjoined, as of different <strong>DNA sequences</strong> in a <strong>transgene construct</strong></td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>Enzyme used in <strong>PCR</strong></td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>target</td>
<td>Region of DNA under detection in an assay</td>
</tr>
<tr>
<td>template</td>
<td>Sample DNA used in a PCR to which a primer anneals</td>
</tr>
<tr>
<td>terminator</td>
<td>DNA sequence regulating gene expression</td>
</tr>
<tr>
<td>threshold</td>
<td>Maximum acceptable level, or minimum detectable level.</td>
</tr>
<tr>
<td>transformation</td>
<td>Transfer of DNA during genetic modification</td>
</tr>
<tr>
<td>transgene</td>
<td>Novel gene introduced by transformation</td>
</tr>
<tr>
<td>Western blotting</td>
<td>Transfer of protein onto a membrane for an immunological assay</td>
</tr>
</tbody>
</table>