

# Detection of GMOs (Molecular)

by

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Analysis of transgenic plants is used today to answer the same questions as ten years ago. Since then, a wide variety of new molecular and biochemical technologies as well as genetic tools have become available. Some of the commonly used technologies and applications are listed below.

## Commonly used analytical technologies and appropriate applications

Technique	Applications
PCR (Polymerase Chain Reaction)	Rapid screening of transformants to focus subsequent analysis Identifying transgenics in segregating populations
Quantitative PCR	Estimating copy number (total) Hemizygote / homozygote discrimination (potentially)
Southern hybridization	Screening for presence/absence of specific sequences Assessing complexity of integration/transgene rearrangements Estimating copy number (rearranged and unrearranged) Estimating whether transgenes inserted at single or multiple loci “Fingerprinting” identity of independent events
Reverse transcription PCR	Transgene expression: accumulation of specific mRNA – can be (semi-) quantitative and used for determining relative levels
Northern hybridization (a)	Transgene expression: quantifying specific mRNA accumulation Estimating mRNA size
ELISA (b)	Transgene expression: rapid screening for presence / absence of specific protein Can be very high throughput Quantifying specific protein accumulation
Western immunoblot	Transgene expression: screening for absence / presence of specific protein Approximate quantitation of specific protein Estimating protein size, evaluating protein processing
<p>The ribonuclease protection can be used in many cases to obtain analogous results. Compared with northern hybridization, it is generally regarded as more sensitive and offers the potential for greater throughput but it cannot be used for estimating RNA sizes.</p> <p>Western immunoassays of protein dot blots can provide some of the advantages of an ELISA as compared with traditional western immunoassays of electrophoresed proteins (e.g. increased throughput), potentially with reduced assay development requirements.</p>	

## **Transgene Presence and Copy Number Determination**

Primary regenerants or putatively transformed plants are immediately subjected to molecular analysis to determine the presence of transgene and its copy number to narrow down the number of transgenic plants that will be studied further.

**Polymerase Chain Reaction.** Polymerase Chain Reaction (PCR) is a technique that is used to amplify the number of copies of a specific region of Deoxyribonucleic Acids (DNA), in order to produce enough DNA to be adequately tested. The steps involve the preparation of the DNA sample, the master mix, and the primers, followed by detection and analysis of reaction products

The DNA is heated, which causes paired strands to separate (single strands now accessible to primers). A large excess of primers relative to the amount of DNA being amplified is added, and the reaction mixture is cooled to allow double strands to form again. Polymerase (like taq polymerase) is added which can read the opposing strand's DNA sequences and extend the primer's sequence by adding deoxyribonucleotides (dNTPs) together in the order in which they pair across from one another – A:T and C:G. After one cycle, more primers and deoxyribonucleotides (dNTPs) are added and the cycle is repeated. The primers bind with the template sequences and with the newly synthesized sequences. The enzyme again extends primer sentences to repeat the replication process. Thus, an ample amount of DNA is synthesized and all of it will be copies of this particular region.

PCR technology is perhaps the most popular method for screening material for the presence or absence of transgene sequences. It is particularly useful for rapid analysis of large numbers of samples. Despite its popularity, PCR has several inherent characteristics that limit the conclusions that can be drawn. First, a positive PCR shows only that sequences homologous to the primers exist in a sample in close proximity for a product to be made. The result does not indicate whether the template DNA source is the intended sample material or is due to contamination. Neither does it indicate whether the template DNA is integrated onto the plant genome. Information about the sequence outside is not provided. However, when the products are analyzed using electrophoresis, verification of product size can be made. Southern analysis of product can confirm that the sequences in between the primers is as expected, but this step is time consuming and thus is rarely carried out.

The extreme sensitivity of PCR results in the potential contamination and false positive results. According to Register (1997), attention to primer design, experimental design and logistics (how and where the work is carried out) is sufficient to minimize the occurrence of false positive results.

**Southern hybridization.** In 1975, E.M. Southern published an important new procedure that allow investigators to identify the locations of genes and other DNA sequences on restriction fragments separated by gel electrophoresis. The essential feature of this technique is the transfer of DNA molecules separated by gel electrophoresis to a nitrocellulose or nylon membrane. The DNA is denatured either prior to or during transfer by placing the gel in an alkaline solution. After transfer is completed, the DNA is immobilized on the membrane by dyeing or ultraviolet-induced cross-linking to the filter. A radioactive DNA ("probe") containing the sequence of interest is then hybridized or annealed with the immobilized DNA on the membrane. The probe will anneal (form a double helix) only with DNA molecules on the membrane that contains a nucleotide sequence complimentary to the

sequence of the probe. Non-annealed probe is then washed off the membrane, and the washed membrane is exposed to x-ray film that detects the presence of the radioactivity in the bound probe. After the autoradiogram is developed, the dark bands show the position(s) of DNA sequence that have hybridized with the probe (Gardner et al, 1991).

Southern blot analysis is one of the more powerful tools available for molecular characterization of transgenic plants (Sambrook et al, 1989; Dansmuir et al, 1988; Bers et al, 1985). Depending on how restriction digests are designed, information regarding the complexity of transgene insertion(s), the number of transgene copies present, the integrative status, and the number of chromosomal sites where the transgene(s) have been inserted, can be obtained.

For analysis of copy number and integration complexity, digests should be designed to excise the entire DNA fragment from which information is desired. Most often, this will be a plant transcription unit since it contains both the coding sequence and accompanying regulatory sequences. The vector DNA used for transformation should be digested and analyzed alongside sample genomic DNA to provide the necessary controls for evaluating rearrangements and copy number. Samples of bands of a different size from the control band are considered rearranged. However, this analysis does not characterize rearrangements.

Southern blot analysis can be used to provide evidence for transgene integration, including insertions at multiple sites, although misleading results can be obtained. The analysis is most straightforward when the material being analyzed is produced using *Agrobacterium*.

## **Transgene Product Analysis**

Transgene expression studies typically examine either protein or ribonucleic acids (RNA) accumulation. While such analysis does a great deal to characterize transformants, the results do not directly address their transgenic status. Thus, correlating expression with stable transformation is appropriate for all and the best characterized transformation systems.

**Ribonucleic Acids (RNA) analysis.** In most cases, analysis of transgene expression focuses on proteins or other end product since the desired phenotype results from accumulation of a specific protein. When protein analysis is not possible, analysis of RNA transcripts is often used. The techniques such as reverse transcription (RT) PCR, northern analysis, and the ribonuclease protection assay (RPA) can be used to measure ready-state RNA levels.

**a. Reverse transcriptase PCR.** The thermostable polymerases used in the basic PCR process require a DNA template and, as such, the technique is limited to analysis of DNA samples. Yet numerous instances exist in which the amplification of RNA from an organism is preferred. In order to apply PCR methodology to the study of RNA, the RNA sample must first be transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. This process is called reverse transcription (RT), hence the name RT-PCR (Promega, 1996). RT-PCR can be used as a quick and relatively high throughput screen for the presence or absence of a specific transcript. The advantages of RT-PCR, as compared with northern analysis or RPA, are the small amount of materials needed, the high sensitivity

and throughput, and ease of sample preparation. Unlike the northern analysis, however, RT-PCR results provide no information about transcript size.

**b. Northern blot analysis.** If DNA molecules can be transferred from agarose gels to nitrocellulose or nylon membranes for hybridization studies, RNA molecules can also be separated by agarose gel electrophoresis and could be similarly transferred and analyzed. These RNA blots are called northern blots in recognition to the fact that the procedure is the mirror image of the Southern blotting technique (Gardner, et al, 1991) with the gene coding sequence as the probe.

**c. Ribonuclease protection assay.** On the other hand, the molecular characterization of specific genes often includes experiments that assay for the presence and accumulation of particular transcripts. The methods used to assay for these transcripts involve the transfer of cellular RNA to a support matrix followed by hybridization with a specific probe. The presence of transcripts is then detected by autoradiography. The ribonuclease protection assay (RPA) avoids the use of a solid support matrix as well as the uncertainties associated with the blot methods. It is a molecular titration of cellular RNA with excess single stranded probe. The procedure consists of a series of hybridization reactions with equal amounts of probe but increasing amount of input cellular RNA. Because the probe is present in excess, a plot of radioactive probe driven into hybrid (as assayed by nuclease sensitivity) versus the input of cellular RNA is linear, and the amount of probe hybridized simply reflects the amount of cellular RNA used. Given an experimentally determined value for the slope, the number of transcripts in an RNA population complementary to a specific probe can be determined. Its objective is to provide a sensitive assay system with which to measure the absolute prevalence level of particular transcripts (Lee and Costlow, 1987).

**Immunoassays.** Antibodies are widely used to detect and identify antigens in a variety of complex mixtures. These immuno detection methods may be divided into two general categories. The first category includes solution-based techniques, such as enzyme-linked immunosorbent assays (ELISAs), immunoprecipitation and immunodiffusion. The other category of immunodetection method is based on the samples that have been immobilized in a solid support, such as membrane to include Western blots and dot blots.

**a. Western blots.** In Western blot analysis in general, antigens immobilized on membranes are detected with antibodies in a three-step process. First, the primary antibody, an IgG directed against the antigen in question, is added to bind potential antigenic sites. In the second step, a secondary antibody-enzyme conjugate, which recognizes general features of all IgGs (anti-IgG), is added to find locations where the primary antibody is bound. The enzyme (AP or HRP) conjugate to the secondary antibody and catalyzes a colorimetric reaction in the third step, when the appropriate substrate is added resulting in the deposition of colored substrate on the membrane at the reaction site. This color provides a visual indication of potential primary antibody recognition. Antigens can be immobilized (“blotted”) on nitrocellulose or polyvinylidene fluoride (PVDF) membranes by one of several methods, depending on the requirements of the experiment. In all cases, the ability to detect a given antigen will depend on the amount of antigen per unit area of the membranes and on the characteristics of the primary antibody (Promega, 1996).

**b. Enzyme-Linked Immunosorbent Assay (ELISA).** ELISA provides sensitive and quantitative detection of specific antigens or antibodies. This is popular due to its sensitivity, safety, versatility, and common availability of the required reagents and equipment. A variety of ELISA formats can be employed, depending on the needs of the researcher. However, most commercially available ELISA are based on the antibody sandwich format. The sandwich ELISA generally requires two antibodies that are directed against a particular antigen. One antibody is passively adsorbed (coated) onto the surface of the wells of an ELISA plate. The wells are then “blocked” with a nonspecific protein solution to keep background levels low. The samples containing the antigen in solution are then added to the wells and incubated for a sufficient amount of time for the antigen to bind to the antibody immobilized on the plate. After washing the wells to remove the unbound reagents, the second antibody is added to the well. This second antibody binds with the immobilized antigen completing the sandwich. The second antibody is detected with an enzyme conjugate specific for the second antibody. Alternatively, the second antibody itself can be labeled for subsequent detection. When the enzyme substrate is added to the wells in the final step, the conjugate enzyme, and therefore the antigen, is added by observing the colorimetric, fluorescent or chemiluminescent reaction products in an appropriate ELISA plate reader (Promega, 1996).

Western immunoassays and ELISA have largely complimentary strengths and weaknesses. Advantages offered by the former over ELISA include less assay development (ELISA development can be nearly as much art as science) and the ability to assess protein molecular masses (Register III, 1997). In addition, acceptance results can often be obtained using relatively impure antibody preparations. However, western immunoassays take more time than ELISA in approximating the quantitative results.