AGRICULTURAL MATERIALS

Immunoassay as an Analytical Tool in Agricultural Biotechnology

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Immunoassays for biotechnology engineered proteins are used by AgBiotech companies at numerous points in product development and by feed and food suppliers for compliance and contractual purposes. Although AgBiotech companies use the technology during product development and seed production, other stakeholders from the food and feed supply chains, such as commodity, food, and feed companies, as well as third-party diagnostic testing companies, also rely on immunoassays for a number of purposes. The primary use of immunoassays is to verify the presence or absence of genetically modified (GM) material in a product or to quantify the amount of GM material present in a product. This article describes the fundamental elements of GM analysis using immunoassays and especially its application to the testing of grains. The 2 most commonly used formats are lateral flow devices (LFD) and plate-based enzyme-linked immunosorbent assays (ELISA). The main applications of both formats are discussed in general, and the benefits and drawbacks are discussed in detail. The document highlights the many areas to which attention must be paid in order to produce reliable test results. These include sample preparation, method validation, choice of appropriate reference materials, and biological and instrumental sources of error. The article also discusses issues related to the analysis of different matrixes and the effects they may have on the accuracy of the immunoassays.

During the past decade, a large number of genetically modified (GM) crops have been developed using methods of modern biotechnology. These GM or "biotech" crops exhibit unique agronomic traits such as herbicide tolerance or insect resistance, which offer significant benefits to farmers. The development of GM crops is accomplished by using molecular biology methods, essentially by the integration of novel DNA sequences into the plant genome. The new DNA encodes for the expression of the novel protein in the targeted tissue, resulting in the unique agronomic trait. The novel protein and DNA are present in many parts of the plant, in harvested grain, and often in the food fractions prepared from grain.

The enthusiasm with which many farmers around the world have embraced this technology is illustrated by the fact that in the year 2004, 9 years after their introduction, 29% of the total global acreage of soybean, corn, cotton, and canola, the 4 principal biotech crops, were derived from biotech varieties (1). This shift of global agriculture towards biotech varieties, however, has not been supported by all elements of society. Some resistance to the development of this technology does exist. In response to these differing levels of

acceptance of the use of this technology, several countries have adopted regulations requiring that foods prepared from GM ingredients be labeled as such. However, labeling of foods is necessary only when the concentration of GM material in a food ingredient measures above a specified threshold concentration (%GM). The adoption and implementation of such laws can have significant consequences to global commerce in agriculture, food, and feed. Meeting these global market requirements for GM compliances is further complicated by the fact that each country has different regulations, including different GM ingredient thresholds for labeling and different methods of testing (2).

Establishing compliance with GM food labeling laws is dependent on the availability of test methods capable of determining the presence and or concentration of GM ingredients in food or bulk consignments of agricultural commodities such as seed and grain. GM content can be determined by methods that detect either the novel protein or the inserted DNA. Numerous methods for detection of GM DNA using the polymerase chain reaction (PCR) have been developed, and the fundamental elements of PCR analysis for detection of biotech products have recently been reviewed (3).

Detection of the novel proteins produced by GM crops relies almost exclusively on the application of immunoassay technology (4–8). Commercial immunoassays are available for most of the GM crops on the market today and have been used in a variety of large-scale applications, including testing for unapproved events, determining GM content (%GM) ensuring compliance with non-GM labeling requirements, and confirming the presence of high-value commodities in an identity preservation (IdP) program.

Principle and Test Formats

Principles of Immunoassay

Immunoassays are based on the reaction of an antigen (Ag), e.g., transgenic protein, with a specific antibody (Ab) to give a product (Ag-Ab complex) that can be measured. There are many different immunoassay formats, and the choice of format is dependent on the target molecule and application. For macromolecules, such as proteins in AgBiotech applications, enzyme-linked immunosorbent assay (ELISA) and lateral flow device (LFD) are the most commonly used test formats. LFDs are designed for qualitative yes/no testing. ELISA can be used as either a qualitative or a quantitative assay. Two other test formats used in AgBiotech product development and seed quality testing are Western blot and immunohistochemical staining. The Western blot is primarily a qualitative analytical method and is particularly useful in protein characterization because it provides additional information regarding molecular weight. Immunohistochemical staining is used to determine the location of the expressed proteins in the plant. In this section, the key components of immunoassay, antibody, antigen, and the common assay formats are described and discussed.

Antibodies

Immunoassays use antibodies as detecting reagents. Antibodies are glycoproteins produced by specific cells of the immune systems of animals in response to stimulation by a foreign substance. The foreign substance that elicits the production of a specific antibody is referred to as an antigen. The attribute of an antibody that makes it useful as a reagent in a diagnostic kit is its capacity to bind specifically and with high affinity to the antigen that elicited its production.

Polyclonal and Monoclonal Antibodies

There are many antibody-producing cells within the body of an animal, and each cell makes a single unique antibody with its own unique sensitivity and specificity. Within the body of an individual animal, it is possible to have different antibodies that bind to different antigenic sites, so-called epitopes, on the same antigen molecule. An antibody reagent that contains many different antibodies, each reactive with different epitope on the same antigen and produced by different cell, is called a polyclonal antibody. In contrast to polyclonal antibodies, a monoclonal antibody is produced by a single cell (hybridoma cell) and binds to a single epitope. The hybridoma cell is created in vitro by fusing an antibody-producing lymphocyte from an immunized animal with a myeloma cell and can be cultured indefinitely as a means of producing large quantities of monoclonal antibodies.

Polyclonal antibodies are relatively easy and inexpensive to prepare in a relatively short time frame (e.g., 3–4 months); however, the quality of the antibody reagent varies from animal to animal, and it is necessary to prepare large pools of qualified reagent to support long-term commercial production of uniform product. Monoclonal antibodies require greater time (e.g., 6 months) and skill to produce and are more expensive to develop than polyclonal antibodies; however, once the desired monoclonal antibody-producing hybridoma has been isolated, it can be cultured over long periods of time and serve as a virtually unlimited source of uniform, highly specific reagent. In applications where discrimination between very closely related molecules is required, it may be more advantageous to use a highly specific monoclonal antibody reagent. Conversely, in an application designed to detect all the members of a family of closely related molecules it may be more advantageous to use a polyclonal antibody reagent. The selection of one reagent type over another is dependent on the desired performance characteristics of the test method.

Sensitivity

The interaction between antibody and antigen involves binding of the antigenic epitope to the complementarity determining region (CDR) of the antibody. The strength of binding between the 2 is referred to as the affinity of the bond. In general, the greater the affinity of the bond, the greater the sensitivity (lower limit of detection; LOD) of the test method. Sensitivity of a test method is determined not only by the

affinity of the antibody for the antigen, but by factors such as protein expression level, extraction efficiency, and the size of the sample taken for analysis. It is common to detect concentrations of 0.01% in many other products, e.g., Cry9C protein in StarLink® corn (9) and CP4 EPSPS in Roundup Ready soybeans.

Specificity

An antibody binds only to the antigenic determinant that elicited its production. This specificity enables the development of test methods that require minimal sample preparation. The ability of antibodies to bind to nontarget molecules that have secondary chemical and structural similarity to the target antigen is referred to as cross-reactivity. Cross-reactivity can result in false-positive responses or overestimation of antigen concentrations. Unless a transgenic protein has been deliberately engineered in a way that its amino acid sequence is essentially identical to a protein present in the sample extract, cross-reactivity of an antibody to a component of the sample or other GM crop is highly unlikely and almost never a significant issue.

In the case of AgBiotech, some test methods have been described as cross-reactive because they cannot discern the difference between different GM events expressing the same protein. For example, 3 major corn events express the Cry1Ab protein from *Bacillus thuringiensis* (Bt; MON810, BT11, and event 176) and antibodies reactive to Cry1Ab detect all 3. Technically, the antibody is not cross-reactive because it detects the same epitope on the Cry1Ab molecule expressed in each of the different corn events. However, some view the overall test method as cross-reactive because it cannot discern the difference among 3 corn events.

Antigens

Antigens are substances that induce a specific immune response resulting in production of antibodies. Antibodies are capable of discerning even subtle differences in antigen structure, and therefore it is important to immunize animals with antigens that are as close as possible in structure and chemical composition to the form of the antigen as it exists in the sample to be detected.

Plant-Derived Proteins

The ideal antigen for immunization would be the actual GM protein as it is expressed in the plant. However, purification of the novel protein from plant tissue can be difficult and may result in undesirable modifications to the target protein. In addition, purification rarely results in 100% pure protein and immunization of animals with such preparations results not only in the production of antibodies to the target protein but to the contaminants as well. Polyclonal antibodies made from these preparations typically exhibit high background and poor sensitivity. Monoclonal antibodies can be developed using this approach as long as specificity to the target protein is demonstrated during the antibody screening and selection process. In practice, purified plant-derived proteins are rarely used for the purpose of making antibodies for AgBiotech tests.

Microbial-Derived Proteins

A more common approach to making antibodies to GM proteins is to express and purify the protein of interest from an alternate host such as *Esherichia coli* using genetic engineering techniques. Although the amino acid sequence of these recombinant proteins may be the same as the plant-produced protein, post-translational modification may be subtly different, and purification may result in modifications to the secondary and tertiary structure (e.g., denaturation). As long as antibodies that bind to the plant-produced protein with sufficient sensitivity and specificity can be isolated, then differences in structure between plant-produced and microbial-derived proteins are not an issue. Commercially available ELISA kits for Cry1Ab, Cry1Ac, Cry1F, Cry9C, and PAT and CP4 EPSPS proteins are all based on antibodies generated from microbial-derived proteins.

Synthetic Peptides

In certain instances where purified or recombinant antigens are not available or are exceedingly difficult to obtain, or where antibodies to very specific amino acids are desired, short peptides (haptens) conjugated to carrier proteins may be used to develop antibodies. However, peptide antibodies may be more reactive to denatured forms of the protein and therefore often find better utility in Western blot (5).

Enzyme-Linked Immunosorbent Assay

In GM protein detection, a commonly used immunoassay format is the antibody sandwich ELISA. In this format, a 96-well microplate is coated with a primary antibody to capture target antigen in the sample. A secondary antibody, conjugated to an enzyme such as horseradish peroxidase, is used to detect the presence of the bound antigen, which results in a sandwich of the analyte between the primary and secondary antibodies. A typical commercial ELISA kit contains an antibody-coated microplate, an enzyme-conjugated secondary antibody, standards, controls, and enzyme substrate for color development, washing buffer, and sample extraction buffer. The ability to quantify the antigen in the sample is one of the advantages of microplate ELISA. In addition, the 96-well format allows for analysis of many samples in a single assay and the assay can be completely or partially automated depending on the budget of the individual laboratory.

Standards

To determine the concentration of an antigen in a sample, standards correlating to known concentrations of the antigen are used to produce a dose-response curve. The standard curve and the assay response from the samples are used to determine the antigen concentration. The material used to make the standards should yield a response that correlates to the actual
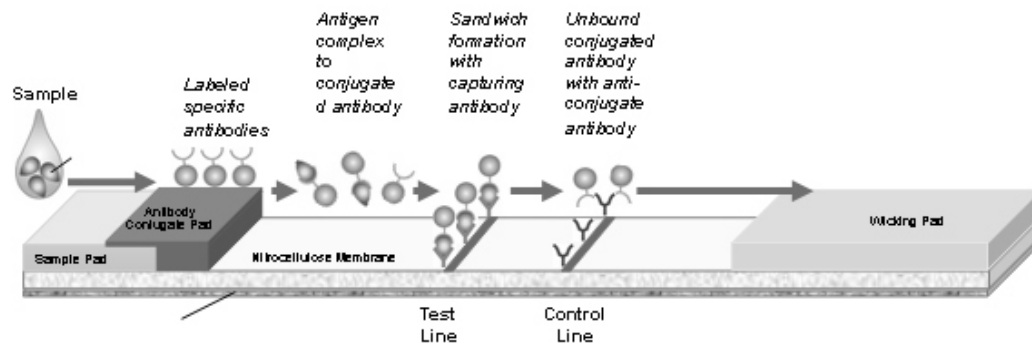
Figure 1. Diagram of a lateral flow device (LFD). The lateral flow device consists of nitrocellulose membrane on a backing material with antigen-specific capturing antibody in a test line and an anticonjugated antibody in a control line. The detection antibody is conjugated to gold and dried onto a fiber pad (gold pad). Optimized buffers necessary for the test performance are provided by the sample pad. The strip also contains a wicking pad made up of fiber and provides necessary wicking for the fluids to move through the membrane. When a positive sample is applied to the strip, the target antigen in the sample first binds to gold-labeled antibody and flows through the membrane, forming a sandwich with the capturing antibody present in the test line. This results in formation of a visible line, and the result is interpreted as positive. The excess gold-labeled antibody further moves and binds to antidetection antibody in the control line and the second line develops. This second line, often termed as control line, serves as an internal control. The device is negative if only the control line is present.

concentration of antigen in the sample type and assay conditions specified by the test procedure. Microbial recombinant proteins, which contain a similar or identical amino acid sequence and immunoreactivity as the plant-expressed protein are often used as ELISA standards. Uniform preparations of actual samples (such as ground corn) having known concentrations of GM proteins may also be used as standards. Regardless of the type of material, it is essential that the standards are of known consistency and account for the effect of sample matrix and sample preparation procedure on antigen reactivity.

Controls

Controls are reagents and specifications that validate each ELISA run. Reagent controls may be different from standards. Every ELISA test, qualitative or quantitative, should include known positive and negative controls to ensure assay validity. Typical controls specify limits for background, assay response to a known concentration, quantitative range, and variability between replicates.

Lateral Flow Devices

LFDs are used for qualitative or semiquantitative detection of antigens. LFDs for the detection of GM proteins use antibodies in the same sandwich immunoassay format used in ELISA, except that the secondary antibody is labeled with a colored particle such as colloidal gold rather than an enzyme as a means of generating a visible signal. A typical LFD is shown in Figure 1 and consists of a sample pad, a conjugate pad, a nitrocellulose membrane, and a wicking pad assembled on a thin plastic backing. The device contains one mobile, labeled detection antibody located in the conjugate pad and

2 stationary capture antibodies located upstream in the nitrocellulose membrane.

The first capture antibody is specific for the GM protein and is immobilized at the test line. The second capture antibody is specific for the unbound detection antibody and is immobilized at the control line. The sample pad facilitates filtration of unwanted particulate material from the sample. Once the liquid sample is added, it moves laterally through the device by capillary action. When the sample reaches the conjugate pad, the GM protein is bound to the labeled antibody. The antigen-antibody complex continues to flow through the strip until it reaches the test line where the immobilized antigen-specific antibody (first capture antibody) binds the GM protein and forms a sandwich of GM protein between the detection and capture antibodies. The accumulation of many colored particles at the test line results in a visible line, indicating the presence of the target antigen. Liquid continues to flow up the strip, and any unbound labeled detection antibody is captured at the control line by the second capture antibody. A colored line in the control zone demonstrates that the liquid flowed through the test zone and that the device is working properly; a missing control line indicates an invalid result. If no GM protein is present in the test solution, only the control line appears and the result is negative. If 2 lines appear, the result is positive. Figure 2 gives an example of the 3 different readings; a positive, a negative, and an invalid result, obtained by an LFD.

Applications

Testing During GM Crop Development

Life science companies use results from immunoassay test methods to influence key decisions from early product
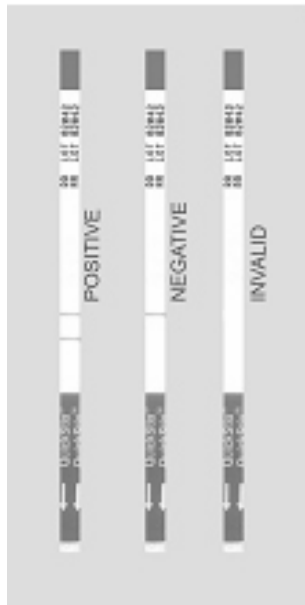
Figure 2. Visualized test results by LFD. Development of the control line within the stipulated reaction time indicates that the LFD has functioned properly. Any LFD that does not develop a control line should be discarded and the sample retested using another strip. If the sample extract contained target protein, a second line (test line) will develop on the LFD, within the stipulated reaction time. The results should be interpreted as positive for the target protein. Any clearly discernible test line is interpreted as positive. If no test line is observed after stipulated reaction time has elapsed, the results should be interpreted as negative, meaning that the sample contained either no target protein or target protein present at levels below the LOD.

discovery through production, sales, and support of GM products. Figure 3 illustrates some of these key decision points and where the various immunoassays are used. Quantitative, semiquantitative, and qualitative methods are all used for data gathering at various stages in the process.

Gene Discovery and Transformation

One of the most common immunoassay methods used during early stages such as gene discovery and transformation in the GM plant product development process is Western blot. Western blots are usually qualitative or at best semiquantitative, and the main objective of the testing is usually to determine whether the target protein is present or absent. One advantage of the Western blot is that the protein is identified by molecular weight as well as reactivity with the antibody. The disadvantages of Western blots are that they are not quantitative and are relatively costly and slow.

Other common applications at this early stage of transgenic plant product development include immunohistochemical staining, used to determine the location of the expressed proteins in the plant. In immunohistochemical staining, the protein of interest is decorated with the detection antibody and is visualized under microscope. The antibodies used in this application can be labeled with gold (5 nm), or with enzymes

for visual observation under compound microscope or with florescence that can be observed using special fluorescence microscopes. In GM applications, a typical protocol involves the preparation of a plant tissue sample and staining the target antigen in the sample. The preparation of samples involves fixation and embedment of plant materials in a resin to preserve the antigens, followed by sectioning of the material with a microtome, resulting in ultrathin cross sections of the plant tissue. The tissue sections are then glued to a glass slide using polylysine or other suitable commercially available glues. The labeling or staining involves blocking the sections with a protein blocker followed by incubation with labeled antibodies. The resulting Ag-Ab complex is viewed with the aid of a microscope. When enzyme-labeled antibodies are used for detection, further washing steps are necessary, and the signal is generated by substrates that produce a colored insoluble precipitate that can be visualized with a regular compound microscope. The use of immunohistochemistry allows the localization of antigens in the plant tissue and can be used for semiquantification in comparison to other samples. However, this technique requires special skills and equipment, antibodies that bind to the fixed antigens, and it is time-consuming.

Event Selection, Backcrossing, and Hybrid Development

Later stages of transgenic plant product development include the processes of event selection and backcrossing. Many events (defined as unique insertions of new DNA into the plant genome) are generated and characterized in order to select the best events for commercial development. Backcrossing is the process of breeding to integrate the best event(s) into the companies' best commercial germplasm. Because the number of samples to be analyzed increases dramatically during event selection and backcrossing, an
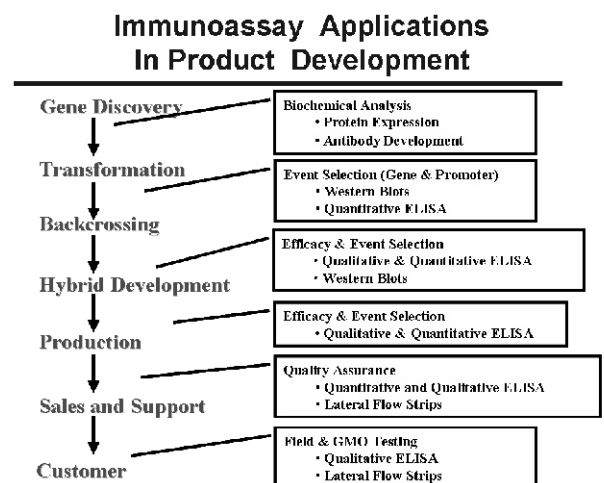
Figure 3. Key decisions during many steps in the development of transgenic plant products are influenced by data generated from immunoassay analyses.

ELISA is usually developed and validated for the target protein at this time. Qualitative ELISAs are used to determine whether or not the transgene is expressing the protein of interest. In the example shown in Figure 4, quantitative ELISA is used to determine the protein expression level. Protein expression level is then compared with insect bioassay results demonstrating the efficacy of this protein.

Registration

The application of quantitative immunoassays is crucial for generating regulatory data packages for all agencies globally. For all AgBiotech products that express a novel protein in quantities sufficient to generate insect-resistance or herbicide-tolerance, different plant tissues need to be analyzed for the amount of this newly expressed protein. These analyses include leaves, roots, shoots, and other plant tissues in order to demonstrate the efficacy and the safety of AgBiotech products. For specific products, additional data to determine the amount of protein in soil, rhizomes, insects feeding on plants that express this new protein, and predators of those insects may be necessary (10–13).

The scientific literature is full of examples of such data and their use in risk assessment and safety evaluation. For example, the lack of any significant impact of Bt-pollen in larvae of the monarch butterfly was studied extensively using relevant exposure data generated by immunoassay (14).

Other examples include demonstration of advantages of AgBiotech plants with respect to food or feed application or regarding impact on the environment. The quantitative determination of the amount of newly expressed proteins in different typical growing regions for at least 2 years and in different tissues allows the assessment of a possible exposure and thus plays a crucial role in any risk assessment (15).

To ensure the accuracy of the analytical results, the developers of AgBiotech products need to validate their immunoassays on all applicable matrixes prior to their intended use. Furthermore, the validation can be a requisite for approval by regulating authorities.

Production and Product Support

Seed producers frequently use ELISA and LFDs to define the level of seed quality. For example, a seed lot may be tested to confirm that 98% of the seeds in a particular lot contain the value-added trait of interest. ELISA and LFDs are also used by seed producers to test for the adventitious presence of unintended events during scale up and production. Most seed quality testing at the seed producing companies is qualitative in nature.

Testing Within the Supply Chain: Seed, Grain, Food, Feed

Testing against a threshold is an intrinsic and important aspect of seed quality control. Seed production companies and grain handlers may apply testing to ensure the presence or absence of a given GM trait within a population, lot, or consignment. Testing thresholds vary depending on the markets, crops, regions, test points within a process, and desired purity. When testing samples comprise discrete particles, such as seed and grain, it is possible to use qualitative methods with statistical sampling plans to determine if GM content is above or below specified thresholds with a high statistical confidence. Although the confidence in the result is high, limited information is available regarding how far above or below the threshold the true concentration is. Threshold methods using rapid LFDs for detecting GM grain have been developed for use in field applications (16, 17). Grain sampling procedures and
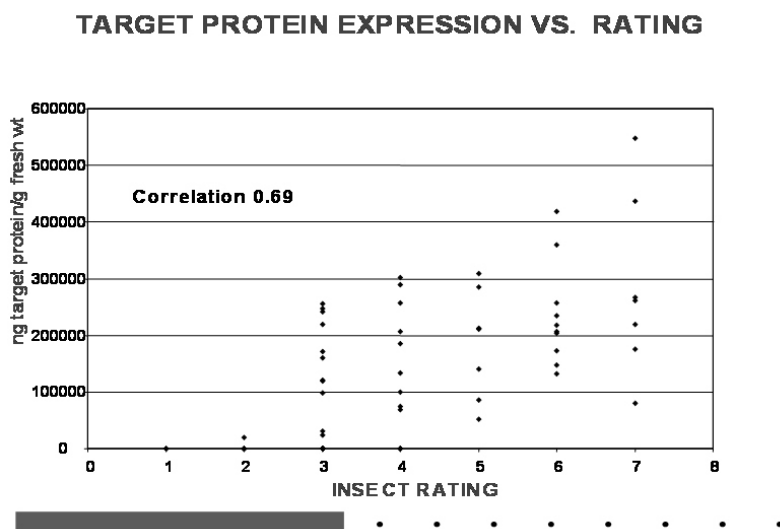

TARGET PROTEIN EXPRESSION VS. RATING

Figure 4. This figure shows a positive correlation between the amount of protein as determined by quantitative ELISA and efficacy of the protein in an insect bioassay. This analysis has demonstrated that the protein may be of interest for further evaluation as an insect-resistance trait.

statistical considerations are described on the U.S. Department of Agriculture, Grain Inspection, Packers and Stockyard Administration (USDA/GIPSA) Website (18). Statistical considerations in seed purity testing have been published by Remund et al. (19) and Laffont et al. (20).

Testing for Unapproved Events

Until now, the largest single application of tests for GM substances is the detection of insect-resistant StarLink corn expressing the Cry9C protein from Bt. Once this trait was found to be commingled in the food supply chain, the U.S. government and industry moved quickly to implement a threshold testing methodology using an LFD to identify and channel StarLink-containing grain into feed distribution channels.

A testing methodology was used that could detect 0.125% StarLink in bulk consignments of grain with 95% confidence (4). Representative samples were taken using standard grain sampling procedures specified by the USDA/GIPSA. Test strip performance was certified by GIPSA according to the U.S. Federal Grain Inspection Service Directive 9181.2, and kits meeting this criteria received a GIPSA certificate of performance (COP). Only kits holding a 9181.2 COP can be used for testing and certification of StarLink in the official inspection system according to the directive 9181.1 (21).

Since September 2000, approximately 7.8 million rapid strip tests have been used to detect and control the distribution of unapproved StarLink corn, and these so-called strip tests have demonstrated that their simplicity, accuracy, and reliability make them amenable to field use and have enabled testing on a very large scale in a rapid and cost-effective manner. Even though a GM product is no longer available on the market, e.g., StarLink and other transgenic crops from several companies that have been phased out and replaced by second- and third-generation products, the use of rapid protein tests for their presence is still common.

Testing for Non-GM Labeling

Some grain markets may require that an agricultural commodity or food substance be "non-GM". In general, this means that the consignment is originated from conventionally bred grains, and systems have been used to minimize the accidental commingling of this grain with GM grains. An illustration of this is the sourcing of non-GM soybeans. Consumers desiring to purchase non-GM soybeans can purchase these beans from originating countries that have not commercialized the production of GM soybeans or through specialty programs that use rapid threshold strip test protocols to reject consignments that contain GM soybeans. For example, LFDs have been in large-scale use in Brazil since the 2002 harvest to ensure that consignments of beans are non-GM (22).

Testing for GM Content

In certain applications it is useful to know the concentration of GM ingredients in a sample. The most common example of this application is the analysis of food fractions and finished foods to determine if the %GM content is in compliance with GM food labeling laws. In these instances, quantitative, laboratory-based methods, such as the ELISA, are required. For reliable results, these methods must be rigorously validated to perform to defined specifications with the sample type under analysis.

Grain Handling and Processing Applications

The food and feed supply chain uses immunoassay technology at several stages. Testing begins as early as the planting of seed in fields and extends through handling and processing, and in some cases, is applied to the finished food ingredient. Figure 5 shows a simplified supply chain from the farm to the end customer, indicating where immunoassay technology can most beneficially be applied. The most common form of test applied in the food and feed supply chain is the LFD. These strip tests are used to detect the presence or absence of GM events, and in combination with sampling statistics, to test lots for the presence of events at specific thresholds (e.g., <5% GMO).

Farm-Level Testing

The need for identifying GM grains at the farm level is mainly concentrated in specialty programs (e.g., non-GM) where the presence or absence of GM grains or the presence or absence of specific GM event(s) is a contractual requirement in the program. As such, tests are useful tools in IdP programs. In general there are 2 main applications on the farm: field surveys and bin testing.

In some IdP programs, survey of the field for the presence of GM grains before harvesting of the grain is required. In this case, samples are removed from the field in a specific sampling pattern to assess the level of GM material in that particular field or region. LFDs are used to determine the
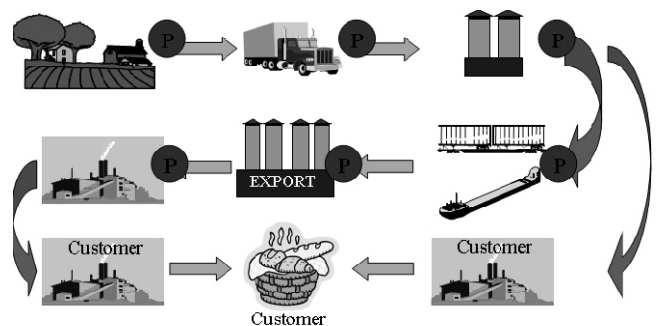
Figure 5.   Potential testing positions for protein-based test methods. Protein-based testing (LFD and ELISA-based) are generally used at the front end of food and feed supply chain in locations requiring rapid test results for channeling of grains. As pictured, protein-based method use is generally restricted downstream as the impacts of processing on the proteins can eliminate the performance capabilities of protein tests.

presence of GM grains or plants. Based on these tests results, fields can be qualified early for specific specialty programs.

LFDs can also be used on the farm to confirm the GM status of storage bins before delivery to the elevator or to confirm the GM status of bins for record keeping. Most farms have some level of storage on the farm, especially if they produce both GM and non-GMO crops. LFD can be used as an additional verification of the GM status of grains to confirm on-farm records. In this case, producers can draw samples from a specific bin and quickly assess the GM status of the bin.

Delivery to the Elevator

LFDs are used extensively at the elevator by the food and feed supply chain, as this is usually the first point of contact between the producer and the grain handler. This test provides a useful tool to the supply chain because it is a way to verify the conformance of the grain for specific programs before it is commingled with larger consignments. Generally grain is screened for (*1*) the presence of specific GM events, which may be restricted from the programs, and (*2*) the %GM content in the grain to see if the specific lot of grain being delivered is within the specification for the program.

In the case of restricted events, these are generally events that are not approved for use by the specific end-use customer. For example, grain exported to another country may have events that are approved for use in the country of origin but may not yet have completed the regulatory approval process in another country. Because these events cannot be screened for zero tolerance, in some cases there are specific protocols agreed to between the buyer and seller on how these events will be tested. In other cases, the testing is conducted at the discretion of the grain buyer at the elevator.

For programs with %GM specifications, there is a limitation on the amount of adventitious presence (AP) of GM grains that can be present in a consignment of grain and still be accepted in the IdP program. In these cases, a consignment of grain is most often tested to determine the level of AP in the grain before delivery. In this scenario, a representative sample is removed from the consignment and tested for the presence of specific events at a predetermined threshold using LFDs and statistical analysis or using strip readers. Based on the result of this analysis, the consignment is either accepted into the program and combined with the rest of the grain or rejected from the program.

Barge and Rail Testing

Grain that is destined for export generally needs to move from the growing area to an export position near the coasts, which are usually located long distances from the major growing areas of a country. In several of these exporting countries, grain is preferably moved to an export position by rail or waterways, which are more economical ways to move grain. Grain is most often transferred from storage facilities onto unit trains or by barge to the export position. During this transfer of the grain there is an opportunity to collect a sample that is representative of the consignment (e.g., a barge, a railcar, a unit train, etc.), and in some cases this sample is tested to see if the consignment is in specification before it arrives in the export position. Because there are generally several days between loading and arrival at the export location, these samples can be tested with additional methods beyond LFD, such as ELISA or PCR.

Testing of Raw Material for Food Processing

Large amounts of grain are also delivered directly to processing facilities for conversion into food and feed ingredients. Depending on the scale and volume of a particular facility, some programs may originate and control for specialty grains, such as GM content. In addition to truck delivery, these facilities may also receive grain by barge or rail. The use of LFDs at these locations is similar to that described for delivery of grains to the elevator. In some cases, a representative sample is pulled from the consignment and analyzed via protein testing prior to processing.

Food Fractions and Food Processing

The amount and nature of protein present in food and food ingredients may be significantly affected by processing steps. Food processing may consist of a series of (sometimes elaborate) processes to transform raw agricultural commodities (e.g., soy beans, corn kernels) into the desired food or food ingredient. For example, for corn the first processing step separates the different tissue types within a corn kernel (endosperm, germ, and coat) and feeds them into different process streams. The germ is used for oil extraction, the remaining meal is typically used as animal feed, and the endosperm is further processed into starch.

The changes that occur to a protein during processing may lead to denaturation. Denaturation of food proteins has been defined as a process in which the spatial arrangement of polypeptide chains within the molecule is changed from the native protein to a more disordered arrangement. Loss of its tertiary structure and truncation are among the key outcomes of protein denaturation. Thus, heating, steaming, drying, exposure to nonphysiological pH or salt concentrations, excessive agitation, freezing, and thawing, can result in denaturation. Microbial processes used to produce specific food characteristics can alter endogenous proteins, e.g., milk proteins are altered during the production of fermented products (yogurt, cheese). During fermentation proteins change form, are often altered in solubility, and may become fragmented.

Protein testing for GM content can be applied to processed food or feed; however, care must be taken to ensure that the test applied is validated and fit for purpose. Typically, protein testing for GM content is applied to minimally processed products (corn, soy meal, flour, de-fatted soy flakes, soy milk, tofu), but specific applications have been developed for highly processed products like toasted soy meal and protein isolate (17) and for the analysis of Cry9C in food and food ingredients (23, 24).

The critical issue for testing for GM content in processed foods by immunoassay is that the antibody must react to the

antigen as it exists in the processed food. During validation it has to be ensured that known samples, representative of the process conditions, can be successfully analyzed. The emphasis should be less directed on the composition of the food ingredient and more on process conditions (especially heat treatment profiles) applied during production.

Testing for the Presence of High-Value GM Commodities

Although most of the testing in commodity grains has been to restrict GM grains either at zero tolerance (unapproved events) or at specific thresholds (approved events), in some applications GM commodities are valued over non-GM, and tests are used to make certain that buyers receive the added value that they are paying for. Currently, the most common application of this type of testing is the quality control of cottonseed production. Indeed, this was the first commercial application of testing for GM commodities and was introduced in 1997. Cotton farmers buy GM seed for their unique agronomic characteristics, and seed producers test a specified number of individual seeds from each lot to ensure that the percentage of GM seed is very high and meets the product claim. This procedure is executed on samples taken from trucks waiting to unload and uses an LFD strip test methodology and sampling regimen designed to yield very high levels of statistical confidence in the result. Today, there are a wide array of GM cotton varieties expressing 1, 2, and even 3 unique GM traits, and single strip tests capable of detecting all 3 traits independently are in routine use. This same approach is likely to be used in the future as value-added GM grains begin to be commercialized and IdP programs are used to manage their increased value through the supply chain.

Immunoassay technology has been used extensively to test for GM commodities on a large scale in a variety of applications. The correct application of these tests is dependent on factors such as biological variability, sampling, sample preparation, method performance, calibration, reference materials, and method validation. The fundamental aspects of the technology and factors affecting performance are discussed below.

Sampling Considerations

Simply stated, a sample is meant to be a representative subset of material that was derived from a lot (25). A simple random sample is one selected in a process in which every possible sample from a lot has an equal chance of being selected, and such a random sample will produce an unbiased estimate of the measurement for the lot being tested. However, samples are selected in a random manner, and a sample test result will, therefore, rarely produce an identical value since there is variability among samples taken from the same lot. The technologies used for protein testing on agricultural commodities include ELISA, Western blotting, and LFDs as previously discussed (22, 26, 27). Generally, these technologies test only a subsample from a larger sample because the bulk sample in most cases is very large (tons) and

the test can accommodate only small samples (grams). Thus, sample preparation and analytical methods are 2 significant sources for error that must be considered in evaluating an analytical measurement. Developing appropriate sampling plans can help minimize errors attributable to sampling and ensure that the sample is an accurate representation of the lot. Finally, statistics and probability can be used to estimate the likely range that a sample deviates from true lot content, as long as the sample is representative of what is in the lot. More details to the statistical considerations can be found elsewhere (19, 20).

Sampling Steps

When protein-based assays are used to test for the presence of transgenic events in grains and oilseeds, a number of sampling steps may occur: (*1*) sampling of consignment of seed/grain to obtain bulk sample; (*2*) sampling of bulk sample to obtain the laboratory sample; (*3*) subsampling the laboratory sample to obtain a test sample; (*4*) grinding the test sample and sampling the ground meal (flour) to obtain an analytical sample; and (*5*) sampling a portion of the analytical sample that results from extraction of the ground meal sample to obtain the test portion.

Although all steps in creating the test portion impact the accuracy and precision of the overall test result, the variability reported for many test methods often includes only the variability of the analytical method or portions of the sampling protocol. Because each sampling step contributes to total analytical error, it is important to design a sampling strategy that will result in a suitably representative test portion and take into consideration particle size, extraction efficiency, matrix effects, limit of detection, and range of quantitation.

Particle Size and the Test Portion

Particle size of a sample can affect the ability of the analyst to accurately characterize the sample (28, 29). The equipment and detailed procedure used to grind a sample is an integral part of the total method and must be rigorously specified. The effects of particle size are manifested and become apparent at the limit of detection of the analytical method. In GM testing, the final test portion typically contains target proteins in the concentration range of parts per million or parts per billion. For a test portion of a fixed size, the finer the sample is ground, the greater the number of particles in the test portion. If the number of particles is insufficient, then the method has unacceptably high variability at the limit of detection and can result in an increased rate of false negatives. Increasing the size of the test portion can significantly reduce these effects. The effect of sample size on the precision of ELISA measurements is given in Figure 6.

The effect of number of particles in the test portion can also be seen when designing sampling plans for testing large particles such as seeds and grains. To detect a 0.01% lot concentration with 99% probability requires a sample size of 46 050 kernels. However, a much smaller number of particles (4603) is required to detect 0.1% GM with the same confidence (99%). Even smaller numbers of particles (2995)

are required if the application can accept less statistical confidence (95%). Such a large sample size is not appropriate for most protein or DNA testing methods. A sample that is large can be divided into subsamples of smaller size. Each subsample will require testing, and none of the samples may have a positive result. For example, for a test kit that can detect 1 transgenic kernel in 1000 nontransgenic kernels with a high degree of reliability, but does not have the sensitivity to detect 0.01% concentration, the above sample can be divided into 47 subsamples of almost 1000 kernels each. All 47 subsamples would require testing and would have to test negative to conclude that the lot contains 0.01% or less transgenic material. All calculations in this section were performed with SeedCalc, a software tool (30) distributed by International Seed Testing Association, ISTA Geneva, Switzerland, and is described elsewhere (19, 20).

Method Validation and Proficiency Testing

One of the major challenges of testing for the presence of transgenic events in grains and oilseeds is how to standardize testing procedures. To ensure comparable analytical results by different laboratories, analyses should be performed with validated methods using standard reference materials (3, 31). Method validation is tantamount to estimating the uncertainty of a measurement and describes how a specified method will perform using a well-defined protocol. The validation of methods consists of 3 phases. For in-house experiments, the method is developed and validated to fit its purpose. Then, typically, 2–3 laboratories perform a precollaborative validation of all of the parameters except reproducibility in order to define the applicability of the method. The last phase consists of a large-scale collaborative trial with at least 8–10 laboratories, the main outcome of which is a measure of the repeatability and reproducibility in order to estimate the transferability of methods between laboratories. Several large studies to validate protein-based methods for detection of transgenic events have been performed both in the United States and abroad (9, 32, 33).

Validation Parameters

Method validation is a well established science and international standards have been developed for many fields of analytical measurement (34, 35). The harmonized ISO/IUPAC/AOAC standard (36) was developed for chemical methods and serves as a basis for all analytical method validation. Rigorous standards for immunoassays used in medical diagnostics have been developed and are maintained by such organizations as the Clinical and Laboratory Standards Institute (35). General considerations for methods used to detect GM foodstuffs have been published by Codex (37), and specific international guidelines for validation of immunoassays for the detection and quantification of GM foodstuffs have been developed by ISO and CEN (38). Guidelines for the validation of immunoassays for GM crops and food ingredients have been previously published (27). Guidance for bioanalytical method validation published by the U.S. Food and Drug Administration (2001)
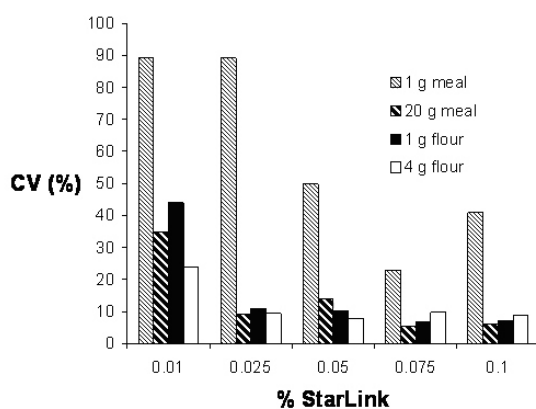
Figure 6. Effect of particle size and sample size on precision in ELISA. As sample size increases, the variability (%CV) decreases. Also, as particle size decreases (from meal to flour), variability decreases. These effects are more pronounced at the LOD of the assay (0.01% StarLink).

includes some specific recommendations for validation of immunoassay. Other sources of validation methodology include EURACHEM's fitness-for-purpose guidelines and the AOAC Official Methods of Analysis Manual. Table 1 summarizes validation parameters for qualitative and quantitative methods of analysis. Stability of the analyte is normally included as part of the validation process, even though it is somewhat method-independent.

Reference to Criteria Set by Validation Organizations–GIPSA

The USDA/GIPSA has a program to verify claims of performance for rapid tests that detect GM events present in grains and oilseeds. To obtain GIPSA certification, the rapid test kit manufacturer submits a data package, including a well-defined testing protocol, supporting its claims for a thorough review of the data by a third party. If the manufacturer's claims are supported by the data, GIPSA performs an in-house verification procedure. If the claims are substantiated, GIPSA issues a certificate of performance to the manufacturer for a period not to exceed 3 years.

The LOD of the method must be inclusive of the lowest expressing commercial variety. No cross-reactivity with other proteins specific for transgenic traits is allowed. Generally, 30 control samples and 30 fortified samples are analyzed using 3 different test lots and tested randomly in a double blind control format (21). Upon analysis, all test results must be 100% correct in order for a rapid test kit to be certified.

EPA Independent Laboratory Validation

During GM crop development, quantitative ELISA is widely used as an analytical tool in field expression, risk assessment, and other related studies for product registration. GM crops expressing insect-resistance traits are regulated by the EPA, and methods for their detection and quantification must conform to EPA guidelines for pesticide residue

Table 1.   Summary and comparison of most relevant method validation criteria necessary for validating quantitative and qualitative methods[a]

Qualitative methods	Quantitative methods
Applicability	Applicability
Specificity	Specificity
Sensitivity	Limit of quantitation
	Linearity or range of quantitation
	Limit of detection
Repeatability	Precision (repeatability)
Matrix effects	Matrix effects
False-positive and false-negative rates	Accuracy
Ruggedness	Ruggedness
Limited reproducibility	Limited reproducibility
Package insert review	Package insert review
Quality policy certification	Quality policy certification
Inclusivity	
Exclusivity	
Percent method agreement	
The 4 performance indicators for qualitative methods are:	The 3 performance indicators for quantitative methods are:
Sensitivity rate	Repeatability
Specificity rate	Reproducibility
False-negative rates	Relative standard deviations
False-positive rates	

[a] Method comparison can include all or some of the listed parameters.

analytical methods (39), including an independent laboratory validation (ILV) prior to submission to the Agency. The ILV is required for enforcement methods by PR Notice 96-1 and must be conducted under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practice standards (40CFR 160). Generally, an ILV study includes at least 1 set of samples or 1 trial, which consists of 2 control samples, 2 control samples fortified at the limit of quantification (LOQ), and 2 control samples spiked at another level defined by the registrant.

Proficiency Testing

GIPSA has organized a proficiency program that provides GMO testing laboratories with the ability to assess their internal capabilities for detecting the presence of transgenic events in grains and oilseeds. This program is designed to improve the reliability and credibility of testing for GM events and is provided free of charge to any organization that has interest in participating.

Challenge samples are characterized and prepared by gravimetrically fortifying finely ground GM and non-GM maize and soybean flour. However, the zygosity of the event material is not always known in the preparation of challenge samples. GM maize/soybeans are blended with GM-free maize/soybeans in concentrations ranging from 0.1 to 5.0%. A library of challenge samples prepared in bulk is dispensed into 20 g aliquots, labeled with a traceable identification number, and stored at 0 C until needed. On a biannual basis, corn and soybean samples are issued to participants to use with their personal in-house methods. The following corn events have been incorporated into the program: T25, CBH351, MON810, GA21, E176, Bt11, NK603, TC1507, and MON863. The soybean samples are glyphosate tolerant (Roundup Ready) or conventional. The USDA/GIPSA Proficiency Program is available to organizations throughout the world, and as of April 2005, over 119 organizations (31 U.S. and 88 non-U.S. organizations) were enrolled in the program. The results from each biannual distribution are summarized and posted on the GIPSA Biotechnology Web page. Organizations used a combination of DNA and/or protein-based methods to report either: (*1*) qualitative results, analyzing for at least one, but not all events; (*2*) quantitative results, analyzing for at least one, but not all events; (*3*) a combination of qualitative and quantitative results, but not analyzing for all events; (*4*) qualitative results only, analyzing for all events; or (*5*) a combination of qualitative and quantitative results, analyzing for all events.

Proficiency programs help organizations identify areas of concern and take corrective actions to improve accuracy, capability, and reliability. Other organizations offering proficiency-testing schemes are the American Oil Chemist Society (AOCS; Champaign-Urbana, IL), the American Association of Cereal Chemists International (AACC; St. Paul, MN), and Food Analysis Performance Assessment Scheme (FAPAS) through its scheme Genetically Modified Materials Analysis (FAPAS-GeMMA®; Central Science Laboratory, Sand Hutton, York, UK).

Reference Materials

General Considerations

Both positive and negative reference materials are required for development, validation, and troubleshooting of antibody-based detection methods as well as for the determination of measurement uncertainty. In addition, they may be used for quality control and quality assurance of a method in the laboratory or in the field. In the method development and validation phases, positive reference materials are used to establish the accuracy, precision, sensitivity, LOQ, and rate of false negatives, whereas negative reference materials are used to determine LOD, specificity, and the rate of false positives. For example, LOD for a test method may be determined by analyzing multiple samples of negative reference material and calculating a mean value. The LOD value is then calculated by (*1*) adding a multiple (usually 2–3) of the standard deviation to the mean of the value of the negative control samples, or (*2*) using the value generated by multiplying the mean value of the negative control samples by 2–3 .

In a completely analogous manner to the discussion of reference materials for PCR-based methods (3), protein-based reference materials of different qualities are appropriate to use in different situations. The different types of reference materials are as follows:

A Certified or Standard Reference Material (CRM or SRM®) is described by a specific certificate, which states that one or more of the property values of the reference material is certified by a procedure that establishes the value's traceability to an accurate realization of the unit in which the property value is expressed. In addition, the certificate states a level of confidence of uncertainty. These types of reference materials are usually issued by National Metrology Institutes such as the Institute of Reference Materials and Measurement of the Joint Research Center of the European Union (IRMM; http://www.irmm.jrc.be) and the National Institute of Standards and Technology (NIST; http://www.nist.gov/) in the United States.

A reference material (RM) is a material or substance having one or more of its properties sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or the assigning of values to materials.

A working standard (WS) is a secondary standard in regular use. This material is equivalent to RM if it is quantified/characterized by comparison with the CRM/SRM.

Quality Standards for Reference Materials

The ISO Guides 30 through 35 describe in quite some detail the applicable general requirements for terms and definitions used in connection with reference materials (ISO Guide 30:1992); contents of certificates and labels (ISO Guide 31:2000); calibration in analytical chemistry and use of certified reference materials (CRMs; ISO Guide 32:1997); uses of CRMs (ISO Guide 33:2000); general requirements for the competence of reference material producers (ISO Guide 34:2000); Certificate of Reference Materials—"General and statistical principles" (ISO Guide 35:1989).

The quality of each reference material is given in a certificate of analysis. A certificate of analysis describes information on the characteristics of the material such as presence and amount of the target material and absence of other possible interfering materials. The certificate of analysis may also specify methods for which the reference material has been validated and is suitable for use.

Concentration of Protein

Both positive and negative reference materials are used to show that the assay is working and its performance has not been compromised by inappropriate storage. The protein concentration of the reference should be appropriate for the range of concentrations and applications for which the assay is validated. If the detecting antibodies can react with more than one GM protein (e.g., to PAT/*pat* and PAT/*bar* proteins), the respective reactivities must be known in order to use the reference material.

Physical Form of the Reference Material

Reference materials for protein detection methods can be either the protein itself purified from recombinant microbes such as *E. coli* , a ground plant matrix (typically leaf or grain), or a processed food fraction. With differences in post-translational processing of eukaryotic and prokaryotic organisms, the relative immunoreactivity of *E. coli* and plant-expressed protein needs to be characterized. The physical form of the reference material determines its suitability for use with any given method. For ground materials, differences in particle size distribution between reference materials and routine samples may affect extraction efficiency of the GM protein and method reproducibility due to sampling error.

Adventitious Presence of Other Protein Analytes

For a reference material to be useful with protein-based methods, it must not be contaminated with other GM events for which there is a known cross-reactivity (e.g., different corn events all containing Cry1Ab), at a concentration above the detection limit of the method. As it is not possible to determine with absolute certainty the complete absence of GM material, negative reference materials will typically state that they contain less than a specified amount of GM material (e.g., <0.1%) at a known certainty (e.g., 99%), which is defined by the methodology used to characterize the reference material.

Genetic Background

It is possible that the expression levels and extraction efficiency could vary for plant matrix reference materials from diverse genotypes of a given crop. As information about the exact variety in the samples for analysis may hardly be available, it is not particularly useful to try to match plant reference materials and test samples. Moreover, no such match is possible if purified proteins are used. However, in evaluating the significance of test results, the potential impact of this biological variation needs to be carefully considered as it will be one of the contributing factors to the overall measurement uncertainty.

Choice of Reference Material

Generally, ground grain/seed or leaf tissue will be used for plant reference material. Seed is the hybrid or inbred material that is planted in the field, and grain is the material that is harvested. Grain may have a different concentration of the analyte compared to seed for hemizygous plants as the trait will be segregating. It is important to realize that the amount of analyte in plant-based reference material may not be an indication of the amount of GM plant material in a sample due to variability of expression of the protein in plants. If the range of expression levels is too great, the amount of the analyte present cannot be used to quantify the % = GM content in processed fractions or food in the same way that quantitative PCR is used. Grain or seeds can be tested using seed pool testing strategies as described earlier.

Proteins purified from *E. coli* and used as reference materials generally are used in ELISA for development of standard curves, determination of LOQ values, and for matrix validation in spike and recovery experiments. Plant reference materials may be of limited use for spike and recovery experiments because the level of expression may be variable and the amount of analyte present must be determined by some other assay such as an ELISA. In addition, the extraction efficiency from plant matrixes is highly dependent on particle size and extraction conditions. The main uses of plant reference materials are as positive and negative controls.

## Sources of Error

General Considerations

Quantitative and qualitative determination of target protein in an unknown sample by immunoassay is solely based on its analytical sensitivity and specificity of antigen-antibody reaction for the analyte of interest. Any substances, processing, or handling that interferes with either antigen or antibody functionality will compromise the accuracy of immunoassay. Many biological factors routinely cause the measurement error in an immunoassay analysis, e.g., antigen form, cross-reactivity, masked or altered antigens, sample treatment (extraction efficiency), matrix effect, variability of protein expression level in plant, and stability of reference antigen and other reagents.

(**a**) *Reference antigen*.—For a validated commercial test kit, the reference antigen (protein) is a pinnacle of quantitative analysis. Unlike small molecule chemicals, macromolecules such as proteins are normally unstable and susceptible to storage conditions. Protein may be degraded or aggregated during storage if the buffer condition is not appropriate. Some proteins may be denatured during freeze-thaw cycles. Thus, the stability of reference antigen needs to be thoroughly evaluated and optimized during method validation. In some cases reference antigen may be derived from microbial sources and may be slightly different from plant protein in its amino acid sequence or with respect to glycosylation, phosphorylation, and/or other post-translational modifications of the target protein.

(**b**) *Expression level*.—Protein expression level in GM plants varies significantly depending on variety, tissue type, plant growth stage, geographic location, and environmental condition. The difference in protein expression levels could be 1 to 2 orders of magnitude. In protein quantitative analysis, such variability has been identified as a major factor for data discrepancy. To obtain an accurate and consistent measurement of target protein in plant tissues, these potential error sources need to be thoroughly considered. Detailed sampling information is very important for data analysis and troubleshooting. In certain immunoassay formats, including LFD, very high expression levels may cause false-negative results due to a hook effect. However, kit manufacturers usually have optimized and validated the assay with high expressing varieties to ensure that this does not occur, thus following the user's guide during testing is required.

(**c**) *Extraction efficiency*.—Extraction of protein from plant tissues with high efficiency and consistency is critical for accurately determining GM protein levels in a sample. The extraction efficiency is dependent on sample type, target protein, sample processing protocol, extraction buffer and protocol, time, and buffer-sample ratios. Even under optimum conditions it is unlikely that all of the target protein will be extracted from the sample. The amount that is extracted is not as important as the consistency of extraction. During method development the extraction protocol for the intended matrixes is validated and specified, and the method is calibrated in a way to correct for extraction efficiency. An extraction method for one sample may not be applicable to another, and, therefore, it is essential that extraction efficiency be assessed prior to analysis when one intends to use the same method for matrixes other than those it was validated for. As demonstrated in Figure 7, even seemingly small differences such as particle size can have a significant impact on extraction efficiency.

(**d**) *Protein stability in samples*.—Any conformational change of an epitope will alter its affinity with antibodies in the assay and thus impact the accuracy of test results. Sample processing may affect protein conformation and thus contribute to method error. Also, proteins may aggregate during storage and this may increase or decrease the immunoreactivity. Storage stability of the GM protein in the bulk sample as well as the extract should be thoroughly investigated during method validation, and suitable conditions for storage should be specified by the method protocol. Fresh samples and sample extracts may be stable for a short time at 4 C; however, it is recommended that they are analyzed immediately unless stability under the storage conditions has been demonstrated. For longer term storage, plant tissues may be lyophilized and stored at –80 or –20 C. Grains, seeds, and processed fractions may be stable for very long periods under conditions of controlled temperature (e.g., room temperature) and humidity (Figure 8).
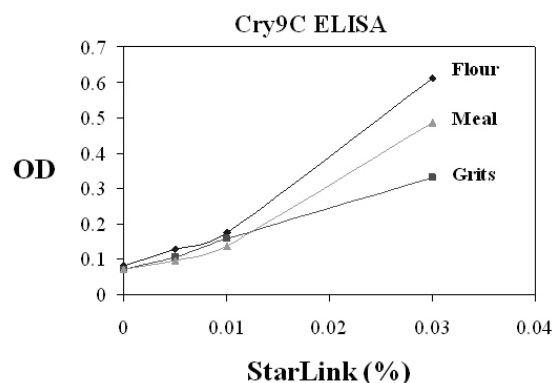
Figure 7. Effect of particle size on extraction efficiency in ELISA. Flour (fine), meal (medium), and grits (coarse) were prepared from the same lot of corn and extracted by identical procedures. More antigen is extracted from samples consisting of finer particles.

(**e**) *Matrix effects*.—There are many types of effects that components of the sample matrix may exert on the test method. Gegenheimer (40) describes 3 types of interferences contributed by plant matrixes: (*1*) plant proteins, which may cross-react in the immunoassay; (*2*) enzymes present in plants such as proteases and oxidases, which may cause protein/antibody degradation or nonspecific bindings; and (*3*) plant secondary products such as phenolics and quinones, which may adversely affect protein extraction, antibody-antigen binding, and enzyme activities. In addition, sample pH, ionic strength, protein composition, viscosity, and particulate matter may all affect method performance. In some cases, matrix effects can be avoided or minimized by simple dilution. Some effects can be controlled with buffers, detergents, proteins, and other polymers. Certain plant tissues like cotton usually have a strong matrix effect due to the presence of a large variety and quantity of phenolics and quinones. To minimize these matrix effects, polymers such as polyvinyl pyrrolidone (PVP) are commonly added to the extracting media. PVP binds to the polyphenols and prevents oxidation of these compounds by polyphenol oxidases (41). Matrix effects are investigated during assay development, and strategies for managing their effects are optimized in the method validation process.

(**f**) *Reagent and kit stability*.—Reference materials are used to calibrate a quantitative method, and equivalence between the reference and the GM plant protein in the sample needs to be established for accurate results. Macromolecules such as proteins are normally unstable and susceptible to change during storage. Protein may be degraded or aggregated if improperly stored. Thus, the stability of reference antigen needs to be thoroughly evaluated and optimized during method validation. Similarly, protein standards, controls, and reagents must be demonstrated to be stable under the conditions of storage and use specified by the test protocol.

Analytical/Instrumental

Lateral Flow Devices

Qualitative interpretation of LFDs gives results in one of 2 answers: positive or negative. The LFD also contains a control line, the absence of which indicates error and renders the test invalid. Assuming that the LFDs are validated and made under strict standard operating procedures (SOPs) and quality assurance, other sources of error could cause either false-positive or false-negative results.

(**a**) *Precipitation of the antibody conjugate*.— Precipitation of gold conjugate during the development of the test can be the cause of false-positive and false-negative results. Colloidal gold conjugates of the type used in most LFDs for detection of GM proteins can be precipitated by extreme salt content or pH (e.g., seed treatments, fermented corn samples). Partially aggregated gold may be trapped nonspecifically at the test line, producing a false-positive result. If the gold is more heavily aggregated, some of it may be prevented from entering the membrane, reducing the signal

and producing a false-negative result. Highly precipitated gold may never enter into the membrane, resulting in an invalid test. LFDs are designed for specific sample types and applications and use specially formulated buffer compositions, either as a liquid to extract the sample or dried in the test device, to control these effects. Use of such tests with samples or procedures that have not been validated may produce false or invalid results.

(**b**) *Stability*.—When LFDs are exposed to inappropriate storage conditions, especially high humidity, false-positive and false-negative results may occur due to aggregation of the colloidal gold and/or loss of antibody activity. LFDs are typically manufactured under conditions of low humidity and packaged with desiccant to keep them dry. As long as the test device is kept dry, a well-constructed LFD can be stable for long periods of time (months to years) at room temperatures and are resistant to degradation of performance at elevated temperatures. During development of the method, performance is validated under the storage conditions specified by the manufacturer.

(**c**) *Impaired flow rate*.—Impaired liquid flow may produce both false-positive and false-negative results and may occur when the extraction ratio of buffer to sample is too low, a sample is too finely ground, or oil or other component of the sample impedes fluid flow. Particulate material can clog the membrane, preventing gold from entering the strip. Partially clogged membranes may cause nonspecific binding at the test line or prevent sufficient sample from washing past the test line, producing a faint false-positive or difficult-to-interpret result.

(**d**) *Operator as source of error*.—These errors are usually associated with improper handling of devices. Most of the LFDs have a limit on the sample volume and the depth at which these are inserted into the sample. If immersed too deeply into liquid, the conjugate pad comes in contact with the
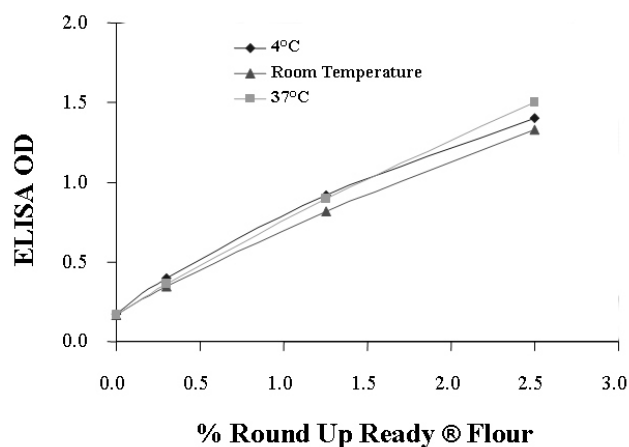
Figure 8. Stability of full-fat Roundup Ready flour ELISA standards. Each data point represents the mean of 11 analyses run over a period of 246 days. The reactivity of the standards stored 246 days at 37 C is the same as those stored at 4 C, demonstrating the stability of full-fat flour stored under these conditions.

sample extract directly, the gold conjugate is released into the liquid, and the test line will be absent or very faint.

When some LFDs are used with highly concentrated plant tissue, chlorophyll may bind nonspecifically to the test line, resulting in the appearance of a light green line (instead of a red line due to the presence of colloidal gold). An inexperienced analyst may interpret such a test as positive.

ELISA Plate Assay

There are many excellent texts covering all aspects of ELISA, including common sources of error (42, 43). Some of the major sources of error are briefly described below and can be classified into 3 categories, namely, errors due to the edge effects and errors due to the instrumentation.

(**a**) *Errors due to the operator*.—As discussed previously, ELISA requires experienced lab personnel to avoid the most common errors, which include not following the manufacturer's instructions, improper pipetting of samples and reagents, and improper or inconsistent washing of microplates.

(**b**) *Errors due to edge effects*.—Humidity and temperature in the lab play important roles in ELISA reactions. As most ELISA procedures require incubation times of over 1 h, dry lab conditions or incubation of ELISA plates close to air conditioning or heating ducts may result in uneven evaporation of liquid from the wells, especially on the periphery of the plate. This can usually be avoided by covering the plate during incubation. Most commercial kits include specific instructions to manage this effect.

(**c**) *Errors due to the instrumentation*.—Automated plate washers and plate readers are often used in ELISA. Automated plate washers may not perform well for washing plates with samples containing particulate matter that may block the washing heads. This results in inconsistent washing, which can have a dramatic impact on many aspects of method performance, including background, sensitivity, accuracy, and precision. With proper maintenance and calibration, plate readers contribute very little error to ELISA methods.

Conclusions

For the detection of products of agricultural biotechnology, immunoassays are frequently used very early in the food and feed supply chain. It is critical that such methods are demonstrated to be reliable and applied according to the manufacturer's recommendation in order to give consistent results in laboratories across the world. This includes the need for a proper validation of the methods. The choice of the appropriate reference material will impact the reliability and accuracy of the analytical results. It is important that analysts pay proper attention to the effect of specific matrixes on the methods. In addition, numerous biological and analytical factors need to be taken into account when reporting results. Immunoassays are valuable and reliable tools for the detection of GM products in seed production and in the food and feed supply chain. When operated within specifications, immunoassays have been proven, in most cases, to be fast, reliable, and economic test methods.

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