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Guidelines for the Validation and Use of Immunoassays for Determination of Introduced Proteins in Biotechnology Enhanced Crops and Derived Food Ingredients

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Recent advancements in agricultural biotechnology have created a need for analytical techniques to determine introduced proteins in crops enhanced through modern biotechnology techniques. These proteins are expressed in plant tissues and may be present in food ingredients. Immunoassays are ideally suited for protein detection and may be used as both quantitative and threshold methods. Microplate ELISA and lateral flow devices are two of the most commonly used immunoassay formats for agricultural biotechnology applications. This paper provides general background information and a discussion of criteria for the validation and application of immunochemical methods to the analysis of proteins introduced into plants and food ingredients using biotechnology methods. It is the result of a collaborative effort of members of the Analytical Environmental Immunochemical Consortium. This collaborative effort represents the combined expertise of several organizations to reach consensus on establishing guidelines for the validation and use of immunoassays. Further, the paper offers developers and users a consistent approach to adopting the technology as well as aid in producing accurate and meaningful results.

Keywords: *Immunoassay, ELISA, introduced proteins, transgenic crops, genetically modified crops, GM food testing, analytical method validation, Analytical Environmental Immunochemical Consortium, AEIC, agricultural biotechnology*

INTRODUCTION

Agricultural biotechnology (ag biotech) is a new technology influencing the agriculture and food industries. A large proportion of major crops currently grown in the USA have been

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enhanced with one or more traits thorough the use of modern biotechnology (about one-third of US corn, one-half of US cotton and one-half of US soybeans) (USDA, 1999). These crops are the result of incorporation of specific agronomic, crop protection or quality traits into plants grown for agricultural production. These plants have been referred to as 'genetically modified organisms (GMOs)', 'genetically enhanced', 'genetically engineered (GE)', 'transgenic plants' or 'biotechnology enhanced plants'. Foodstuffs derived from such crops have been called 'GM, GE, or novel foods'.

Transgenic plants differ from conventional plants because they contain additional genes which produce a corresponding number of new proteins, the presence of which give the plant the desired beneficial traits. A gene for a specific trait is introduced by inserting a new DNA fragment into the plant genome by recombinant DNA techniques. The fragment of DNA (gene) produces a protein which is expressed in the plant tissues. A particular introduced protein may produce a desired agronomic trait in the plant, or it may confer insect, disease, or herbicide resistance, thus improving crop production. A particular introduced protein may also produce a quality trait such as enhanced flavor or nutritional value.

With the introduction of transgenic crops, many applications have emerged for analytical techniques to determine the presence of the resulting proteins in agricultural commodities. These applications are driven by the needs of technology providers, food and commodity distribution channels, and global regulatory bodies. Analytical technology is rapidly evolving and new techniques are continually emerging. Among the most useful techniques for ag biotech applications are immunoassays. Immunoassays offer simple, specific, and sensitive protein detection methods to address a wide range of needs.

As with any analytical method, an immunoassay must be validated for accuracy, precision, sensitivity and specificity. The purpose of this paper is to set forth guidelines for the validation and use of immunoassays for determination of introduced proteins in plant matrices and food ingredients. The focus will be on two immunoassay test formats, microplate ELISA for quantitative testing, and lateral flow devices for threshold testing. The paper will discuss current applications of these tests for ag biotech, differences between quantitative and threshold methods, the validation and use of immunoassays, and the importance of appropriate sampling plans to obtaining meaningful results.

This paper represents the collaborative efforts of members of the Analytical Environmental Immunochemical Consortium (AEIC). The AEIC has provided a forum for technical experts from the ag biotech and diagnostic kit industries to work collectively to reach consensus resulting in these general recommendations and guidelines.

PRINCIPLES AND TEST FORMATS

Immunoassay

In its most generic form, an immunoassay is an analytical method dependent on the specific binding of an antibody with its target analyte. Detection is achieved by the addition of a signal-generating component (reporter label) which gives a response to the target analyte. Many types of labels have been used for immunoassays including radioactivity, enzymes, fluorescence, phosphorescence, chemiluminescence, and bioluminescence. (Gee *et al.* 1994)

Microplate ELISA and lateral flow device test formats are currently the most commonly used for ag biotech applications. These formats often use enzymes and colorimetric substrates to produce a response to the target analyte. The microplate ELISA can be used as a yes/no assay, a threshold assay, or a quantitative assay. Lateral flow devices are designed to give a yes/no result or an indication whether the sample contains the target analyte at or above a stated threshold level.

For detection of proteins, sandwich immunoassays are the most common type of immunoassay employed. Sandwich immunoassays involve immobilization of a capture antibody on a solid phase support, such as on the wells of microplates or on membranes. The solution containing the analyte is introduced and antibody-analyte binding occurs. A second,

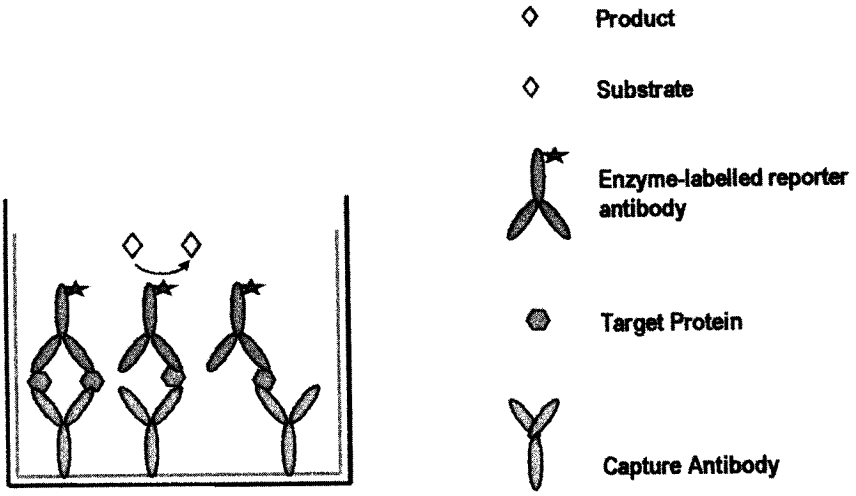

FIG. 1. Sandwich immunoassay.

analyte-specific, labeled antibody is added and it also binds to the analyte, forming a sandwich. For enzyme immunoassays, a colorimetric substrate is added and reacts with the enzyme, producing a colored product. (Figure 1) The magnitude of the color or optical density (OD) is directly proportional to the concentration of the analyte in the sample solution.

Microplate ELISA

The microplate ELISA test is conducted in standard 96-well microplates. A microplate consists of a 12 × 8 grid of wells for test solutions. Microplates are also available as individual 8 or 12 well strips for more testing flexibility. (Figure 2) In microplate sandwich ELISA, analyte-specific capture antibodies are immobilized on the plastic surfaces of the microplate wells. Test solutions and enzyme-labeled reporter antibodies are added to the wells and antibody-analyte binding occurs. Following an incubation period, the unbound analyte and reporter antibodies are washed away, leaving only antibody-analyte-antibody complexes which are bound to the well surfaces. A substrate solution is added which reacts with the enzyme label and the resultant colored product is visually interpreted or measured spectrophotometrically.

A typical microplate ELISA test can be done by a trained analyst in a laboratory in about 1–8 h. Required instrumentation costs about $5000–10 000. Microplate tests can also be used as yes/no or threshold tests as the results can be visually interpreted without instrumentation. For quantitative assays, a standard dose response curve is run concurrently with each sample set, on each microplate, by the addition of standard solutions in a range of concentrations which encompass the stated quantitation range of the assay. Properly characterized standards of the transgenic protein analyte are necessary to prepare the calibration solutions. Because the 96-well format allows for high volume testing, the cost per test well is low (between $0.20 and $10.00 for commercially available kits) and the microplate format is suitable for automation if desired.

Many 'non-commercial' antibodies and immunoreagents exist which are capable of detecting proteins in plants derived from ag biotech. These reagents are often useful for research purposes. Analyses performed for regulatory or commercial decisions (e.g. commodity export) should rely on tests and methods which have been properly validated for

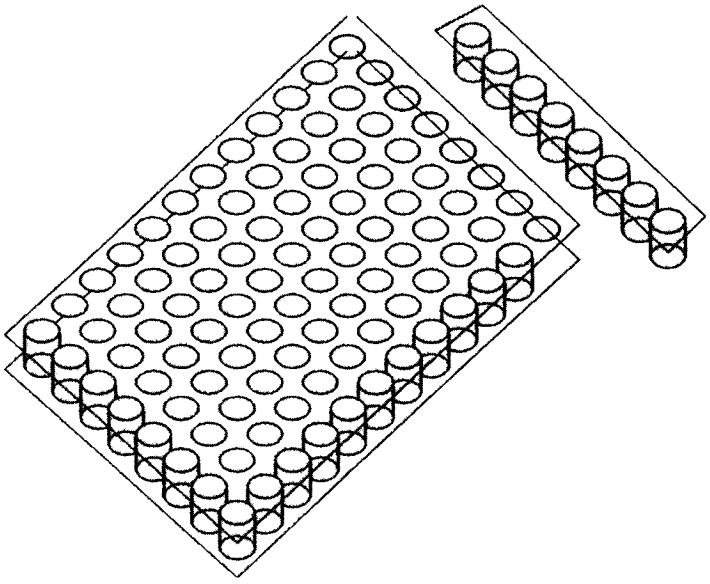

FIG. 2. 96-Well plate for microplate ELISA.

the specific application and for which manufacturing reagents and/or kits have been demonstrated to be reproducible.

Lateral Flow Devices

Each lateral flow device (also called a strip test) is a single unit allowing for manual testing of individual samples. A typical device consists of a reservoir pad, a result window and a filter cover (Figure 3). The device contains an antibody coupled to a colored particle such as colloidal gold or latex which is deposited in the reservoir pad. An analyte-specific capture antibody is immobilized on the membrane (the strip itself). When the strip is placed in a tube or vial containing the test solution, the solution enters the reservoir pad and solubilizes the labeled reporter antibody which binds to the target analyte. This analyte–antibody complex flows with the liquid sample laterally along the surface of the strip. When the complex passes over the zone where the capture antibody has been immobilized, the complex binds to the capture antibody and is trapped, accumulating and producing the appearance of a colored band on the strip. If the result is negative and no analyte is present in the test solution, one band appears in the result window. This band indicates that the liquid flowed properly up the strip. If the result is positive, then two bands appear in the result window (Figure 3).

A lateral flow strip test can provide a yes/no determination of the presence of the target analyte or a threshold (semi-quantitative) result, typically in 5–10 min. Some advantages of this format are that the cost per test is low ($1–6 per strip), it is field portable, it can be done at ambient temperature, and it requires no specialized equipment. Only minimal user training is required. The lateral flow format is appropriate for field or on-site applications.

Additional Immunoassay Formats

In addition to microplate ELISA and lateral flow devices there are a variety of other immunoassay test formats. Another popular format uses magnetic particles as the solid support surface. The magnetic particles may be coated with the capture antibody and the

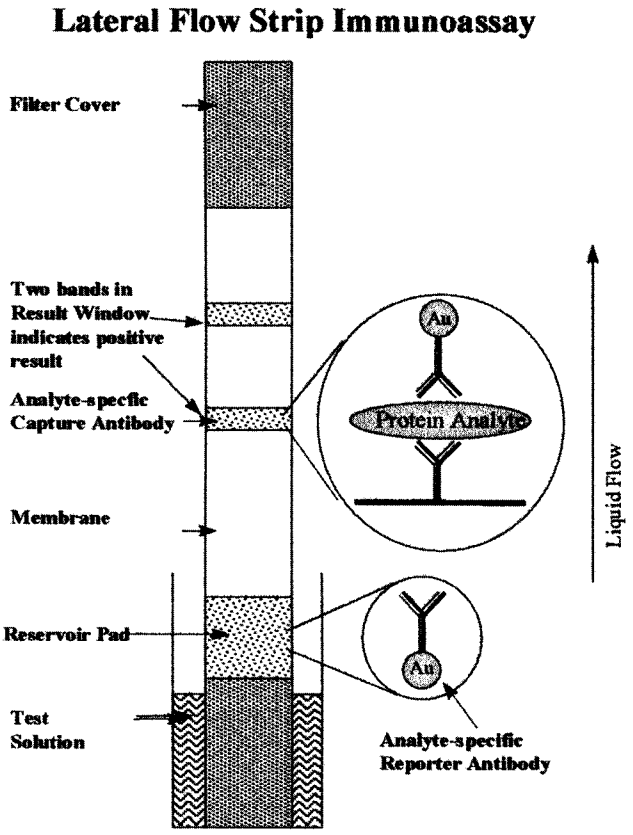

FIG. 3. Lateral flow strip schematic.

reactions take place in a test tube or similar vessel. The particles with bound reactants are separated from the unbound reactants in solution by applying a magnetic field. Two advantages to this format are superior reaction kinetics because the particles are free to move about in the reaction solution and increased precision due to uniformity of the particles. There are also numerous variations on the microplate ELISA formats which include different enzyme tags and substrates, competitive assay formats, and nesting or combining of two steps into one step. Other immunoassay formats that are used in ag biotech product development and seed quality testing include western blots and dot blots, which are particularly useful for analysis of insoluble proteins.

APPLICATIONS OF IMMUNOASSAYS FOR AG BIOTECH

In the area of ag biotech crop development, technology providers include the ag biotech companies, seed companies, food companies, and other research organizations. During all stages of the research, testing and development of ag biotech products both quantitative and threshold testing techniques are used, the choice depending on the specific application (Table 1).

During product development, qualitative and semi-quantitative immunoassays are used for gene discovery, even selection, screening, transformant identification, line selection, plant breeding and seed quality control.

TABLE 1. Applications of immunoassays for agricultural biotechnology (ag biotech)

Ag biotech product development
Screening
Transformant identification
Line selection
Plant breeding
Seed quality control
Regulatory approval
Petition for non-regulated status
Expressed protein levels for regulatory submissions
Generating product characterization data
Assessments of food, feed and environmental characteristics
Determining concentrations for toxicology studies
Obtaining tolerance exemption for pesticidal proteins
Approved product
Product support
Product stewardship
Intellectual property protection
Seed quality control
Grain handling/distribution system
Identification of transgenic products at grain elevator
Testing at other transfer points in supply chain
Identity preservation
Food ingredient testing
Raw agricultural commodities
Processed food fractions

For regulatory submissions, authorities require that expression levels of introduced proteins in various plant parts be determined by quantitative, validated methods. Quantitative immunoassays are used to determine expression data of field material for regulatory approvals. Immunoassays are also used to generate product characterization data; to assess food, feed and environmental characteristics; to calculate concentrations for toxicology studies; and to obtain tolerance exemption for insecticidal proteins.

For the approved ag biotech product, immunoassays are used for product support, product stewardship, intellectual property protection, and seed quality control.

Immunoassays are also useful in the grain handling and distribution system. Threshold assays are most commonly used to test agricultural commodities entering the food distribution channel to ensure compliance with relevant labeling regulations. For example, truckloads of grain are routinely sampled and tested at the grain elevator to ensure that the identity of the grain commodities is preserved.

Some food companies are testing food and food ingredients for the products of genetic modification. Introduced proteins can be found in raw and some processed agricultural commodities. Both proteins and DNA fragments can be denatured or removed during certain processes such as heating, This creates potential difficulties in the analysis of heavily processed finished food products such as oils.

METHOD VALIDATION

Standardized Reference Materials

The standardized reference material consists of the same matrix as the actual agricultural commodity to be tested. For example, if the matrix to be tested is soybean seed, the

standardized reference material would be soybean seed containing a known proportion of transgenic seed. Access to standardized reference materials is important during the development, validation, and use of immunoassays for analysis of introduced proteins in transgenic agricultural commodities. There are transgenic reference materials currently commercially available, but these have been made for only two transgenic events and are in limited supply; therefore for most transgenic crops, analytical labs must rely on the ag biotech companies to supply reference materials. Future changes to regulations and testing needs may change the availability of reference materials.

In the case of commodities such as grain or seed, where the commodity consists of discrete units, it is fairly straightforward to make a reference sample with a known proportion of transgenic material. In other cases, generating reference samples for certain matrices and analytes can be difficult. Stability and uniformity are important considerations. For example, if the plant part to be tested is leaf tissue or pollen, it would be difficult to combine transgenic and non-transgenic material in such a way as to achieve a homogeneous reference sample with a known proportion of transgenic material. The stability of these materials would need to be evaluated under storage and test conditions. In any case, it is useful to have non-transgenic and transgenic plant material available to use as negative and positive controls.

During assay development, the reference material would be used to help select assay parameters which would minimize any interfering effects of the matrix (e.g. non-specific binding of sample components to the antibodies). During validation and use of the assay, the reference materials would be extracted and analyzed alongside the test samples, so the results could be directly compared.

Validation Process and Documentation

Method validation is the process of demonstrating that the combined procedures of sample preparation (extraction, clean-up etc.) and analysis will yield acceptably accurate, precise and reproducible results for a given analyte in a specified matrix. For commercially available immunoassay kits, assay performance is validated by the manufacturer and is documented in the product user's guide.

Part of performing the method validation is preparing a written method. Components of a written analytical method are discussed below. This document should include all of the information needed by an analyst to perform the entire analytical procedure, as well as background information and the method validation data (Mihaliak & Berberich, 1995).

Suggested Documentation for a Validated Immunochemical Method

A. Summary of method. The summary is a condensed version of the method, an overview of the sample extraction and analysis procedures.

B. Analyte. The analyte should be clearly identified and its basic characteristics thoroughly described.

C. Principle of method. An overview of the scientific principle of the method is described here.

D. Analytical procedure.

Materials: A list of all materials, reagents and solutions required to execute the method including instructions on the preparation of any solutions or mixtures and any critical settings on instrumentation.

Methods: In this section a detailed step-by-step description of the procedures is given. Sufficient detail should be included to allow an analyst to execute the entire method using only the included information.

E. Results/discussion. The validation data and a discussion of those results are included in this part of the written analytical method. Accuracy, extraction efficiency, sensitivity, specificity, ruggedness and method limitations are method characteristics that should be described thoroughly in the analytical procedure.

Accuracy: Accuracy is demonstrated by measuring the recovery of analyte from fortified samples and is reported as the mean recovery at several levels across the quantitative range. Ideally, quantitative methods will have demonstrated recoveries between 70 and 120% and a coefficient of variation (CV) of less than 20% for measured recoveries at each fortification level (Mihaliak & Berberich, 1995).

Extraction efficiency: Extraction efficiency is a measure of how efficient a given extraction method is at separating the protein analyte from the matrix. It is expressed as percent analyte recovered from the sample. Since the introduced protein expressed is endogenous to the plant, it can be difficult to demonstrate efficiency of the extraction procedure. There may not be an alternate detection method against which to compare the immunoassay results. One approach to addressing extraction efficiency is to demonstrate the recovery of each type of introduced protein analyte from each type of food fraction by exhaustive extraction, i.e. repeatedly extracting the sample until no more of the protein is detected (Stave, 1999).

Precision: Intra-assay precision describes how much variation occurs within an assay. It can be evaluated by determining the variation (%CV) between replicates assayed at various concentrations on the standard curve and on the pooled variation (%CV) derived from absorbance values in standards from independent assays performed on different days. Interassay precision describes how much variation occurs between separate assays and can be measured by analysis of quality control samples on every microplate. The quality control samples required would consist of two pools of extracts, one extract from transgenic plant tissue and one from conventional plant tissue. These extracts would be stored frozen and a portion would be thawed and assayed on every microplate. Interassay precision could be evaluated over time and expressed as % CV (Rogan *et al.*, 1999).

Sensitivity:

1. The sensitivity of the assay could be defined as the amount of analyte that can be measured by an absorbance reading of two standard deviations above background absorbance (Rogan *et al.*, 1992). The detection limit could be expressed as the lowest dilution of transgenic crop that could be detected when transgenic and non-transgenic crop are combined (Rogan *et al.*, 1999).
2. Limit of quantitation: the smallest concentration of analyte that can be measured in samples and yield predicted concentrations with an acceptable level of precision and accuracy (Rittenburg & Dautlick, 1995). This is commonly defined as a concentration equal to the lowest standard used in the assay.
3. Quantitative range: the lower and upper limits of analyte concentration over which the method yields quantitative results within the stated performance limits. This is determined by the range of analyte concentrations used to construct the standard curve.

Specificity:

1. Cross-reactivity: the degree to which analogs or other molecules bind to the antibodies should be characterized and described in the method.

2. Interferences: the potential for interferences from reagents and labware can be evaluated by assaying extracts from non-transgenic plant material.
3. Matrix effects: if the response of the method is affected by a substance in the final extract other than the specific protein analyte, the non-specific response is referred to as a matrix effect. One way to manage matrix effects is to demonstrate that the analytical method gives identical results with or without sample matrix present in the extract. In this approach, freedom from matrix effects would have to be demonstrated in all matrices for which the assay is to be used. Another approach (although less desirable) to managing matrix effects would be to prepare the standard solutions in extracts from non-transgenic matrix, i.e. matrix-matched standards. This would ensure that any matrix effects would be consistent between the standards and the samples.

Ruggedness: Second party validation is recommended as part of method validation. Experiments which may be performed to establish ruggedness include repeated analysis of a sample or samples on several days and measurement of accuracy and precision in fortified samples using control material from several sources.

Limitations: A discussion of method limitations should be included in the written procedure.

E. Conclusions. Conclusions drawn from validation data are discussed here.

F. Tables and figures. Data generated during the validation should be summarized into tables or figures and included with the methods. This provides new users with information on the methods performance as well as reference data to establish quality control parameters.

G. References. References are given here.

QUANTITATIVE TESTING

Quantitative immunoassays are used to determine levels of the protein analyte in specific parts of the plant (e.g. seed, leaf, root, stalk etc.). Typical applications are given in Table 1. In order to perform a microplate ELISA for quantitative determination of a protein analyte in plant tissue, it is first necessary to obtain a representative sample of the plant material. The sample amount will influence the detection limit or sensitivity of the assay. The analyte is then extracted from the plant material by adding a solvent and blending, agitating, or applying sheering or sonic forces. Typical solvents used are water or buffered salt solutions. Sometimes detergents or surfactants are added. Some proteins require more rigorous procedures like homogenization or boiling in solvents, detergents, salts etc.

After the capture antibody has been immobilized on the microplate well surface, a precise volume of the standard or sample extract solution is added to each well. The analyte in the test solution binds to the capture antibody. The enzyme-labeled second antibody is then added and also binds to the analyte, forming a sandwich. At this point, the well is washed to remove unbound analyte and antibodies, leaving only the antibody–analyte–antibody complex bound to the well surface. A colorimetric substrate is added which reacts with the enzyme label and produces a colored product. The reaction is stopped after a set period of time and the color absorbance at a given wavelength is measured on a photometer. The standard curve is generated by plotting the optical density (OD) on the y-axis (linear scale) against the concentration on the x-axis (log scale) which produces a sigmoidal dose response curve Figure 4.

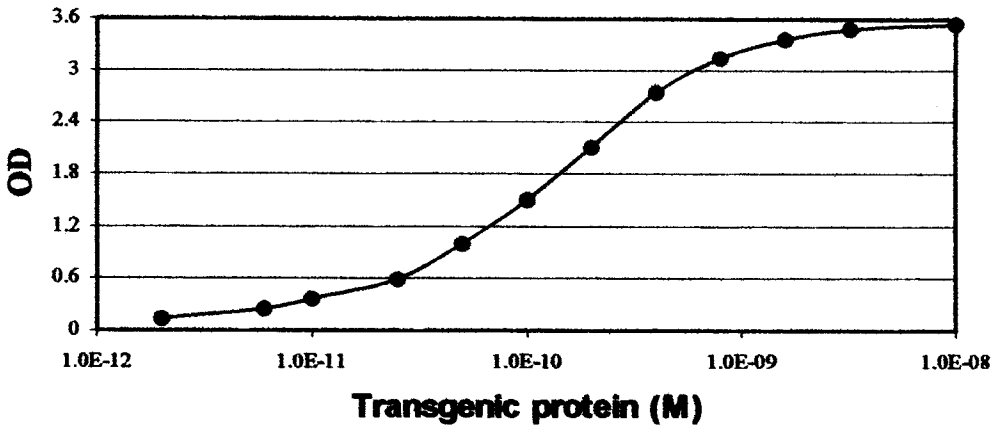

FIG. 4. Standard curve for sandwich ELISA.

To obtain an accurate and precise quantitative value, the OD for the sample solutions must fall on the linear portion of the standard curve. If the OD is too high, the sample solution must be diluted until the OD falls within the quantitative range of the assay. The concentration of the protein analyte in the original sample of plant material is calculated by correcting for any dilution factor that was introduced in preparing the sample for application to the microplate. The initial weight of the sample and the volume of extraction solvent, as well as any subsequent dilutions are used to calculate the dilution factor.

Various assay controls can be employed to demonstrate the performance of the assay. A blank sample such as an empty well or buffered solution can be run in the assay to determine any background response which can be subtracted from sample and standard responses if desired. A negative control sample (i.e. matrix extract solution known to contain no analyte) can be used to demonstrate whether a non-specific response or matrix effect is occurring in the assay. A positive control or matrix extract spiked with a known amount of the analyte can be run to demonstrate accuracy. Standards and samples can be run in replicate to demonstrate precision. Blanks, negative controls, positive controls, fortified sample extracts, standardized reference material extracts, and replicates are typically run on each microplate to control for plate-plate variation.

THRESHOLD TESTING

Lateral flow devices are useful tools for on-site or field threshold testing. This type of testing requires a quick, accurate and cost-effective approach. In order to ensure reliable results, the manufacturer of the lateral flow device must conduct a method validation and provide a description of the performance characteristics of the product in the package insert. If this has been completed there is generally no need for validation studies to be performed by users. Each lateral flow device is an individual stand-alone unit, capable of performing to the standards described in the product package insert.

In order to establish an on-site procedure for threshold testing, the threshold level must first be established. To establish that the lateral flow device is able to differentiate between samples containing transgenic protein above or below the threshold, both a negative reference and a threshold reference containing a known proportion of transgenic grain should be assayed concurrently. The negative reference is a sample of the test matrix known to contain none of the protein analyte and is assayed to demonstrate that the method can distinguish between zero and the threshold level. A sufficient number of these samples are

run to ensure that assay sensitivity is adequate to determine whether the level in the test sample is greater or less than the threshold level. During routine testing of bulk commodity samples, the lateral flow devices would typically be used without running the concurrent negative and threshold reference samples.

Food and Commodity Testing

Immunoassays are useful for determining introduced proteins in food ingredients and commodities. As bulk food and feed commodities move from the farm to the grain elevators and processors, commodities from several sources are often mixed. In order to preserve the identity of the transgenic commodity, it is useful to establish chain-of-custody documentation procedures as well as testing programs. By tracing commodities through the food distribution channel, testing can be limited to key food fractions at critical control points. In this approach, the identity and/or labeling of final foodstuffs could be based on several elements, including the test results of intermediate fractions, the characteristics of the defined process, and the chain-of-custody documentation.

Understanding the food production process is also important in selecting testing locations, frequency of testing and specific test formats. Rapid on-site testing would be necessary in situations where large lots of agricultural commodities are awaiting test results before they can be combined with other lots. For on-site testing applications, the lateral flow device provides rapid turnaround time and only minimal user training is required (Stave, 1999).

Training

The level of training required for personnel conducting immunoassay testing depends on the type of analysis (quantitative vs threshold) and the immunoassay format used (microplate vs. lateral flow device). Quantitative testing using microplate ELISA are usually performed in a laboratory by highly trained, experienced personnel. Threshold or yes/no testing using microplate ELISA where results are interpreted visually would require an intermediate level of training. Threshold or yes/no testing using lateral flow devices may be easily performed by personnel after receiving minimal instruction in the use of the test or simply by following the written instructions provided.

Sampling

Proper sampling is critical in order to obtain meaningful results from any type of analytical assay. The sample must be correct, it must be homogeneous, and there must be a sufficient number of samples to be representative of the whole. Since sampling has the potential to introduce significant uncertainty and error into a measurement, an existing sampling plan should be followed or a proper plan devised with the assistance of a qualified statistician.

When considering an appropriate sampling scheme, it is important to first consider the objective of the test. For example, a plant breeder may take a single leaf punch to quickly determine whether a specific protein has been expressed in an experimental plant. The sampling regime would be more complex for a researcher who wants to determine the expression profile of a specific protein in corn grain, leaves, and stalks for a regulatory study. These studies are often modeled after field crop residue studies for chemical pesticides, and the protocol describes sampling from multiple plants, tissues, growth stages and geographical sites.

Sampling bulk commodities in the food distribution chain requires yet another sampling strategy. For example, while it is possible to determine the exact concentration of transgenic soybeans in a truck, elevator, barge, or ship by testing every bean and calculating the percent positives, this is clearly not practical. However, very low concentrations of transgenic beans can be quantitatively determined by individually testing a large number of beans (e.g. a single positive bean in 10 000 beans tested would suggest 0.01% transgenic content). In this

scenario, the sensitivity of the test need only be sufficient to detect transgenic protein in an individual bean; the detection limit of the analysis would be determined by the number of beans tested.

To minimize the number of tests required to determine an established concentration of transgenic beans, it is possible to combine beans and test a composite sample. The number of beans to be ground in the composite sample is determined by the threshold sensitivity of the test. If the test can detect one positive bean in 1000, then it is appropriate to combine 1000 beans into one test sample (the exact number of beans to composite should be determined by statistical methods). To address the issue of the homogeneity of samples taken from a large vessel, it is important to take samples from various locations within the container. Grain sampling is a routine practice and standard methods can be found in sources such as the USDA Grain Inspection Handbook (USDA, 1995). Ultimately, the optimum sampling strategy is a balance between sensitivity, cost, and confidence.

CONCLUSION

Immunoassays are extremely useful analytical tools for ag biotech applications. The introduction of transgenic crops has created the need for analytical techniques to determine the presence of the resulting proteins in agricultural commodities. Immunoassays provide simple, specific, and sensitive protein detection methods to address the needs of technology providers, the food distribution channel, and global regulatory bodies. Microplate ELISA can provide accurate and precise quantitative results in the laboratory and both microplate assays and lateral flow devices can provide rapid, convenient threshold results for field and on-site applications. Establishing guidelines for the validation and use of immunoassays will help provide users with a consistent approach to adopting the technology and will help users produce accurate and meaningful results from immunoassay methods.

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