SPECIAL GUEST EDITOR SECTION

# Protein Immunoassay Methods for Detection of Biotech Crops: Applications, Limitations, and Practical Considerations

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**Immunoassay methods are available for detection and quantitation of proteins expressed by most biotechnology-derived crops in commercial production. The 2 most common test formats are enzyme-linked immunosorbent assay (ELISA) and immunochromatographic (lateral flow) strip tests. Two ELISA methods, one for Roundup Ready soybeans and one for MON810 Cry1Ab corn, were the subject of large international collaborative studies and were demonstrated to quantitatively determine the concentrations of biotech crops in samples of ground grain. Quantitative ELISA methods are also useful for analysis of processed fractions of agricultural commodities such as soybean toasted meal or corn flour. Both strip tests and ELISAs for biotech crops are currently being used on a large scale in the United States to manage the sale and distribution of grain. In these applications, tests are used to determine if the concentration of biotech grain is above or below specified threshold limits. Using existing U.S. Department of Agriculture sampling techniques, the reliability of the threshold determination is expressed in terms of statistical confidence rather than analytical precision. Combining the use of protein immunoassays with Identity Preservation systems provides an effective means of characterizing the raw and processed agricultural inputs to the food production system in a way that allows food producers to comply with labeling laws.**

The introduction of crops with novel traits derived by modern methods of biotechnology coupled with the advent of laws regulating labeling of foods containing biotech ingredients has resulted in the need for analytical methods to determine the concentration of biotech traits in food substances. An important aspect of food labeling laws is the inclusion of regulatory threshold concentrations above which a food must be labeled. The determination of the content of biotech ingredient can be based on the concentration of novel DNA or protein present in the food substance. The concentration of biotech ingredient based on novel protein content is most commonly determined with immunoassay techniques.

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## Biotech Crops

To understand the application and limitations of any test method used to detect and quantitate biotech ingredients in foods, one must know the types of crops that have been commercialized, their biology, and the extent of their commercial production. The global status of commercialized transgenic crops in 1999 has recently been summarized (1). Currently, 2 major traits (insect resistance and herbicide tolerance) have been engineered into 4 major crops: soybean, corn, cotton, and canola. Protection from insects has been effected through the use of specific genes isolated from the naturally occurring soil bacterium *Bacillus thuringiensis* (Bt). These genes cause the production of specific insecticidal proteins known as Cry proteins. The major use of Bt cry genes for this purpose currently is in corn and cotton. Although there are commercial varieties of corn expressing the Cry1Ab, Cry1Ac, and Cry9C proteins, most commercial acreage of biotech corn expresses Cry1Ab. Four commercial corn events have been approved for import into the European Union (EU; Table 1), and 3 of the 4 express the Cry1Ab protein. The fourth expresses the PAT protein, which confers tolerance to the herbicide glufosinate.

Corn, soybean, canola, and cotton varieties have been developed that are tolerant to a number of different herbicides. All 4 of these crops have been engineered to contain Monsanto's Roundup Ready® gene and express the CP4 enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein. In addition, corn and canola that are resistant to the herbicide glufosinate have been developed, as well as cotton that is resistant to the herbicide bromoxynil. Many other biotech crops have either been approved in the United States or are in various stages of approval. Table 2 lists some additional biotech corn events. Of particular note are Aventis' StarLink corn (event CBH351), which expresses both the Bt Cy9C and PAT proteins, and Roundup Ready corn event GA21. Both of these corn events have been in commercial production in the United States. GA21 is approved for feed and food use in Japan but is not approved for import into the EU. StarLink corn is not approved in either the EU or Japan.

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**Table 1. Major genetic events approved for import into the EU**

| Crop | Event | Company | Protein | Trade name |
|---|---|---|---|---|
| Corn | 176 | Syngenta | Cry1Ab | Maximizer |
| Corn | MON810 | Monsanto | Cry1Ab | YieldGard |
| Corn | Bt11 | Syngenta | Cry1Ab | YieldGard |
| Corn | T25 | Aventis | PAT | Liberty Link |
| Soybean | GTS 40-3-2 | Monsanto | CP4 EPSPS | Roundup Ready |

## Immunoassays

The capacity of immunoassays to quantitatively detect novel proteins in foods derived from biotech crops has been discussed previously (2), and guidelines for validating the performance of such methods have been published (3). Commercial immunoassay methods are currently available for detection and quantitation of biotech crops expressing Cry1Ab, Cry1Ac, Cry3A, Cry2A, Cry9C, CP4 EPSPS, and PAT proteins. The current immunoassays for biotech crops take on 2 different forms, enzyme-linked immunosorbent assay (ELISA) and lateral flow strip tests, and the use of one form or the other is dependent on the particular application. Many of the existing immunoassays were made to support the research, development, registration, and production of biotech crops. Laboratory-based ELISA methods play a major role in selecting cultures of cells or plants that express the novel protein of interest after the initial laboratory genetic transformation. Figure 1 illustrates the configuration of a typical sandwich-type ELISA. Once cells have been transformed, immunoassays are used to select specific genetic events that have optimum levels of protein expression. Protein expression data are required to gain regulatory approval for novel biotech crops, and quantitative ELISA methods are used extensively in this capacity.

During the growth and expansion of a selected event in greenhouses or in the field, immunoassays are used to cull low or nonexpressing plants so that the resulting pool of seed contains a very high percentage of positive kernels. In the commercial seed production process, immunoassays are used as quality control tools to ensure that bags of biotech seed contain very high percentages of the biotech product that the customer is paying for. In several of these applications, it is important to get a result rapidly in the field, and in these situations lateral flow strips are particularly useful. Figure 2 illustrates the configuration of a typical sandwich-type lateral flow strip test. In this configuration, the appearance of 2 lines on the test membrane indicates the presence of the biotech trait, while the appearance of only the top (control) line indicates a negative response. The application of protein immunoassays to testing of grain and processed fractions of grain in support of food labeling laws is a natural extension of the tools used to bring these biotech products to market.

## International Validation of ELISA for Biotech Crops

Two ELISA methods for biotech crops have been the subject of large international collaborative studies designed to assess whether such methods can be used to determine the concentration of biotech ingredients in samples of ground grain in support of food labeling laws. The first study involved an ELISA that detects the CP4 EPSPS protein in Roundup Ready soybeans and was organized by the Joint Research Centre of the European Commission (4). The samples for the study were ground soybean Certified Reference Materials (CRM) prepared by the Institute of Reference Materials and Measurement (IRMM; Geel, Belgium), containing 0, 0.1, 0.5, and 2% by weight of Roundup Ready soybean. These were the same samples that were used in an earlier European ring study designed to evaluate the capacity of a qualitative PCR (polymerase chain reaction) method to detect Roundup Ready soybeans (5).

The ELISA was designed to determine whether the concentration of Roundup Ready soybean was above a specified threshold concentration. At the time of this work, food labeling laws had been promulgated but threshold concentrations were not established; therefore, an arbitrary threshold of 2% was chosen for the study. The results of the study demonstrated that the ELISA could consistently detect Roundup Ready soybeans at 0.3%. In addition, a negative response from the ELISA provided 99% confidence that the sample contained $<2\%$ Roundup Ready soybean, and a positive response provided 99% confidence that the sample contained $>0.85\%$. Al-

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**Table 2. Some biotech corn events**

| Event | Trait | Company | Protein | Status |
|---|---|---|---|---|
| CBH-351 | Insect resistant, glufosinate tolerant | Aventis | Cry9C/PAT | Commercial (withdrawn) |
| DBT418 | Insect resistant, glufosinate tolerant | Monsanto/DeKalb | Cry1Ac/PAT | Commercial (withdrawn) |
| GA21 | Roundup Ready | Monsanto/DeKalb | Modified corn EPSPS | Commercial |
| MON863 | Insect resistant | Monsanto | Cry3Bb | Approval pending |
| NK603 | Glyphosate tolerant | Monsanto | CP4 EPSPS | Commercial |

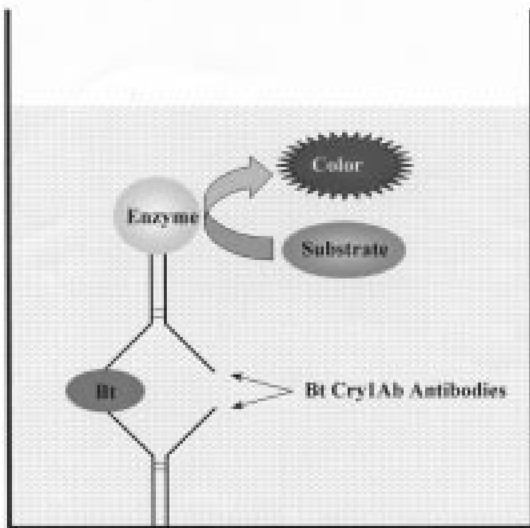


**Figure 1. Bt Cry1Ab sandwich ELISA.**

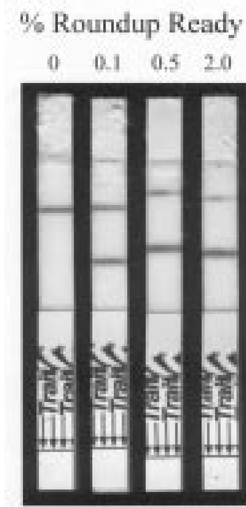


**Figure 3. Detection of IRMM soybean Certified Reference Materials by Roundup Ready strip test.**

though the method was deliberately designed not to determine concentrations quantitatively, the repeatability ($RSD_r = 7\%$) and reproducibility ($RSD_R = 10\%$) of the method for the 2% CRM clearly demonstrated the quantitative nature of the method.

Immunoassay strip tests for biotech traits have not been the subject of formal collaborative studies; however, the utility and power of such tests can be demonstrated with the same CRMs used in the ELISA and PCR studies described above. Figure 3 shows the results of testing approximately 100 mg soy powder prepared by the IRMM, shaken for about 5 s with 1 mL physiologic buffer, and incubated 5 min with a strip test that detects CP4 EPSPS. Two lines can clearly be seen on the strips used to test the 0.1, 0.5, and 2.0% CRMs, indicating a positive response, whereas only the control line is visible on the 0% strip. Considering the time, effort, and expense required to achieve similar results using PCR or even ELISA, strip tests offer significant advantages where simplicity, speed, and cost are primary considerations.

The second ELISA to undergo validation by international collaborative study was designed to quantitatively determine the concentration of MON810 Cry1Ab corn in samples of ground kernels. The study was sponsored by the American Association of Cereal Chemists and a summary of the findings has been published (6). Forty laboratories in 20 countries analyzed 8 samples of whole ground kernels in duplicate. Four of the samples were prepared by POS Pilot Plant Corp., (Saskatoon, Canada), at a pilot scale using standard industry procedures for milling corn flour. The other 4 samples were

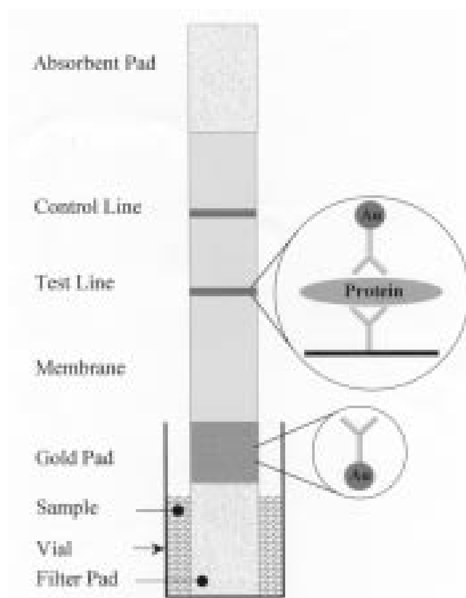


**Figure 2. Illustration of typical sandwich-type immunochromatographic (lateral flow) strip test.**

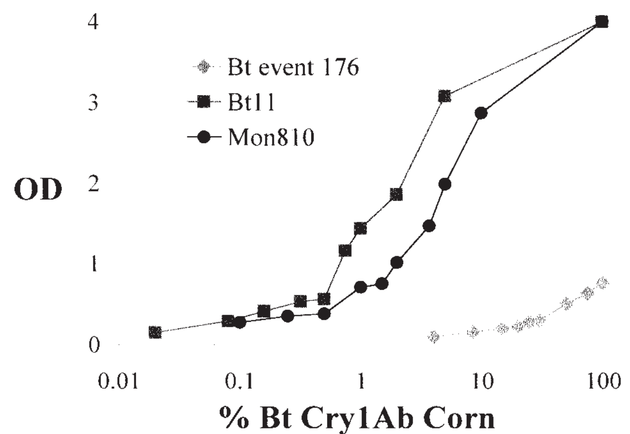


**Figure 4. Reactivity of 3 different commercial Bt Cry1Ab corn events in Cry1Ab ELISA.**

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**Table 3. Protein expression of Bt Cry1Ab in different commercial corn events[a]**

| Event | Form | Seed, μg/g | Leaf, μg/g |
|---|---|---|---|
| MON810 | Truncated | 0.31 | 9.35 |
| Bt11 | Truncated | 4.76 | 20.00 |
| Bt event 176 | Full-length and truncated | <0.005 | 1.00 |

[a] Source: USDA petitions.

prepared as CRMs by the IRMM using a dry grinding procedure of whole kernel corn. The ELISA kit used 4 flour standards of known MON810 concentration prepared from the material made by POS. The robustness of the method was evidenced by the fact that data from all 40 laboratories were included in the analysis. The repeatability (r) ranged from 6.5 to 18.5% $RSD_r$ and the reproducibility (R) ranged from 13.8 to 23.5%. The accuracy of the method for the samples of corn flour ranged from 96.7 to 100% and the accuracy for the CRMs ranged from 113 to 125%. The authors speculate that the apparent over-recovery of the CRMs by the method resulted because the CRMs were ground to a finer particle size than the corn flour standards (determined by using defined mesh sieves), which may have provided greater extraction efficiency.

Although those studies demonstrated the capacity of immunoassays to quantitate biotech ingredients in samples of ground grain, it is essential that the performance of every analytical method be validated with the particular sample intended for the test, including processed food fractions.

## Utility of Protein Immunoassays

All analytical methods have appropriate applications, and a number of factors influence the utility of immunoassays for analysis of food substances containing biotech ingredients. Clearly, if no protein is present in the sample because it was removed during processing (e.g., highly refined syrup or oil), then a protein immunoassay has little utility. If the expression of the protein in the tissue or grain of the biotech crop is extremely low, an immunoassay may not have sufficient sensitivity to detect it at useful concentrations. This is the case with Syngenta's biotech corn event 176. The Bt Cry1Ab protein expressed by this corn event can be detected by ELISA (Figure 4); however, it is at such a low concentration that quantitation around the 1% level is not practical. Although Bt corn event 176 illustrates the limits imposed by sensitivity, the low level of novel protein expressed represents the exception rather than the rule with respect to protein expression in biotech crops. Indeed, for an insecticidal protein to be efficient, the protein must be expressed at relatively high levels; the concentration of Cry1Ab expressed in most Bt corn in commercial production today is at much higher levels. Similarly, the expression of proteins which confer tolerance to herbicides (e.g., Roundup and Liberty) are at concentrations well within the limits of immunoassay technology.

Beyond simple detection, it is necessary to determine whether a food substance contains biotech ingredients above or below specified threshold concentrations, and the capacity of a method to do this is determined by accuracy and precision. The quantitative nature of immunoassay is well established; however, the variability of the final result is dependent on the entire test process, including the variability inherent in the sample and the sample preparation method. The concentration of biotech protein in the tissues of a living plant varies with age and environmental conditions. Also, the concentration of a specific biotech protein, e.g., Cry1Ab, varies between different events expressing the protein (Table 3). Figure 4 shows the response of 3 different Bt Cry1Ab corn events in an ELISA method. In a sample of corn flour where the proportion of these 3 different Cry1Ab corn events is unknown, it would be necessary to develop immunoassays that could differentiate the 3 proteins and quantitate them independently to arrive at a total concentration.

Another factor affecting determination of the concentration of biotech protein in food substances is the reactivity of the antibody reagents for the form of the protein in the sample. Functional proteins have 3-dimensional shapes, or conformations, that contribute to the recognition and binding of the protein by the antibody. Antibodies bind to specific amino acids of the protein, and the degree of binding is determined by the conformation and chemical interaction of the 2 proteins. If an antibody used in an immunoassay binds to the biotech protein only when the protein is in a specific conformation, then denaturation, or unfolding the protein by processes such as heating, can eliminate antibody binding even though the protein is present. This characteristic of antibody binding is well known,

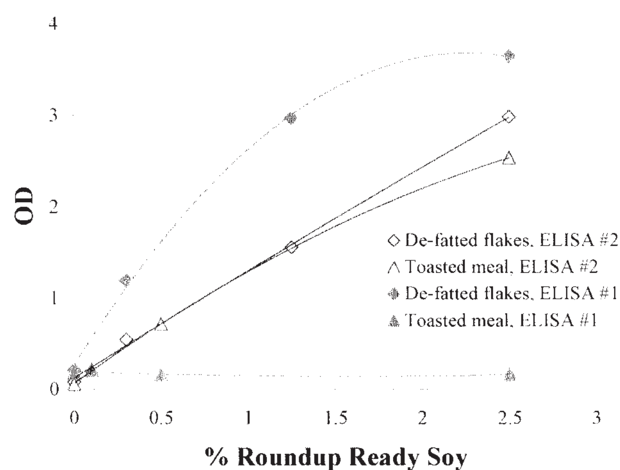


**Figure 5. Reactivity of 2 different ELISAs to processed fractions of soybean. The toasted soy meal was prepared by a process that included heating for 60 min at 100°C.**

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**Table 4. Probability of detecting various percentages of biotech grain using different sample sizes and different numbers of samples[a]**

| | Sample size, kernels | | | | | |
|---|---|---|---|---|---|---|
| | 400 | | 1000 | | 10 000 | |
| | Probability level, % | | | | | |
| No. of samples tested | 95 | 99 | 95 | 99 | 95 | 99 |
| 1 | 0.75 | 1.15 | 0.30 | 0.46 | 0.030 | 0.047 |
| 2 | 0.37 | 0.57 | 0.15 | 0.23 | 0.015 | 0.024 |
| 3 | 0.25 | 0.38 | 0.10 | 0.15 | 0.010 | 0.016 |
| 4 | 0.19 | 0.29 | 0.08 | 0.12 | 0.008 | 0.012 |
| 5 | 0.15 | 0.23 | 0.06 | 0.09 | 0.006 | 0.010 |
| 6 | 0.13 | 0.19 | 0.05 | 0.08 | 0.005 | 0.008 |
| 7 | 0.11 | 0.17 | 0.04 | 0.07 | 0.005 | 0.007 |
| 8 | 0.09 | 0.14 | 0.04 | 0.06 | 0.004 | 0.006 |
| 9 | 0.08 | 0.13 | 0.03 | 0.05 | 0.004 | 0.006 |
| 10 | 0.08 | 0.12 | 0.03 | 0.05 | 0.003 | 0.005 |

[a] Values represent percentage of biotech grain that can be detected with indicated probability when all samples tested are negative. Table supplied by Larry Freese, Grain Inspection, Packers and Stockyards Administration (GIPSA), USDA.

and the approach to developing tests that react with processed foods is to deliberately produce antibodies that bind to denatured proteins by immunizing animals with proteins that have been denatured in a fashion similar to the way the protein will be treated in the food production process.

Figure 5 illustrates the importance of selecting appropriate antibody reagents for the form of the protein to be detected in the sample. The antibodies comprising ELISA #1 were made to recognize the CP4 EPSPS protein in the form found in crop tissue and react well to the protein in defatted soybean flakes but not in soybean toasted meal. The process used to prepare toasted meal includes a heat treatment that denatures many proteins. The antibodies comprising ELISA #2 were selected specifically to react with the CP4 EPSPS protein in the denatured form found in toasted soy meal and react strongly to this processed fraction of soybean. This example illustrates that the choice of a method depends on the specific testing application. Although a particular ELISA may not be appropriate for all food substances, an ELISA to detect Roundup Ready soy meal may have great utility in the animal feed industry. Recent laws banning the use of animal renderings in animal feed to curb the spread of bovine spongiform encephalitis have resulted in increased use of soy meal to replace protein, and producers are concerned about the content of genetically modified soybeans in their animal feed.

## Threshold Testing of Bulk Grain

Recent laws mandating the labeling of food regarding the content of biotech ingredients has led researchers, producers, and regulators to consider quantitating the concentration of novel protein and DNA in finished foods as a way to establish the content of biotech ingredients. However, the number and biological complexity of novel biotech crops that are then processed into tens of thousands of different finished foods makes routine quantitation very complicated and costly. In countries that are major producers of biotech crops, testing is focused more on controlling the distribution of very large quantities of grain rather than analyzing finished foods. To improve efficiency and minimize the cost of testing in this application, the crop must be identified as early in the distribution chain as possible before the grain is pooled into larger and larger containers. A major component of preserving the identity and tracking of biotech grain is complete documentation of the chain of custody as the material moves through the system. A documentation system alone is not sufficient to preserve the identity of biotech crops, and testing is required at critical control points, including the initial point-of-sale when the farmer brings his crop to the local elevator, railcar and river terminals, and ports of export.

A testing system for determining whether a consignment contains biotech grain above or below specified threshold concentrations has been developed which makes use of existing equipment and procedures, U.S. Department of Agriculture (USDA) sampling protocols, and rapid immunoassay strip tests. The statistical probability that a load is above or below the threshold is determined by estimating the probability of detecting one positive unit (kernel, bean, seed), in a large number of units using a simple binomial probability distribution. A number of kernels or beans are taken from a container using USDA sampling protocols, ground together and extracted, and the extract is tested with the rapid test strips. The maximum number of units in the sample is fixed by the procedure so that the presence of a single biotech unit will always

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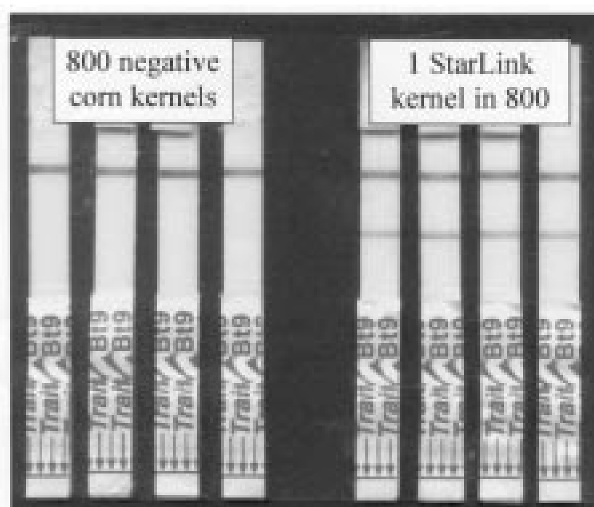


**Figure 6. Threshold testing for StarLink Bt Cry9C corn using strip tests.**

result in a positive test response. For this approach to work, there must be only 2 outcomes of the test, positive and negative, and the accuracy of the procedure should be as close as possible to 100%. The maximum number of units that can be ground together and tested in the procedure is determined by the expression level of the biotech protein in the grain and the intrinsic sensitivity of the test strip, but the sensitivity of the entire procedure is essentially unlimited and determined by the number of independent samples analyzed from the load.

Similar to quantitative analytical methods where the analyst interprets concentration values in light of precision with a stated level of confidence, this threshold approach allows the analyst to interpret test results in terms of statistical probability. If an analyst tests 2 samples containing 400 kernels each and both tests are negative, then there is a 99% probability that a load containing 0.57% biotech grain would be detected. The limit of detection of the method can be improved by increasing the sample size and the number of samples tested. If 5 samples of 10 000 kernels each were tested and all were negative, then there is a 99% probability that the load contains <0.01% biotech grain (Table 4). This threshold method of analyzing grain is not exclusive to immunoassay but can be applied to any test method designed to yield yes/no results with very high accuracy.

## Implementation of Testing—StarLink Cry9C Corn

This type of testing is being implemented on a large scale for StarLink Cry9C corn in the United States. StarLink corn was developed by Aventis CropScience, expresses the Bt Cry9C insecticidal protein, and was approved by the U.S. Environmental Protection Agency for animal feed but not for human food consumption. The conditional registration for animal feed proved unfortunate in that the U.S. grain-handling and distribution system currently does not segregate corn based on intended use, and StarLink was found in food products, including taco shells and tortilla chips. This forced food producers and retailers to issue product recalls and prompted Aventis to withdraw the corn from the market and offer to buy back any StarLink grain that farmers produced but could not sell. The program to buy back StarLink included testing by using Cry9C strip tests (Figure 6). To understand the scope of distribution and to control the movement of StarLink in the system, large grain distribution companies implemented testing regimens throughout the supply chain. The USDA evaluated the strip test and threshold testing method, certified that the performance met the claimed specifications, and implemented a testing program according to USDA Directive 9181.1. The U.S. Food and Drug Administration initiated a field program to collect and test samples of processed corn by using the strip tests, and samples that were positive were confirmed by PCR. In addition to using the strip tests to identify and control the distribution of grain within the United States, the United States and Japan have agreed to a testing protocol based on the strip tests and threshold testing to ensure that StarLink corn is not exported to Japan.

## Identity Preservation and Threshold Testing

It is apparent that testing for biotech grain and food ingredients after they have already been mixed with nonbiotech ingredients is highly inefficient and dramatically increases the costs associated with testing. Indeed, given the number and complexity of different biotech crops and test procedures, it is very difficult to quantitate biotech ingredients in an unknown sample of a food substance, and the difficulty increases the closer the sample is to being a finished food. This complexity is increasing with each new biotech crop introduced into the market, and testing regimens must be able to accommodate this increasing complexity. Identity Preservation (IP) is a system that establishes and preserves the identity of a biotech crop from seed to finished food, including testing at critical control points and documentation along the entire chain of custody. In contrast to testing unknown samples, an IP system tests samples of known identity; under these conditions the number of potential variables is dramatically reduced and the analysis is greatly simplified. Although desirable in some applications, food labeling laws do not mandate quantitation, requiring only determination that a biotech ingredient is above or below a specified threshold. Threshold testing within an IP system provides all parties with a means for controlling raw and processed agricultural inputs to the food production system so that there is high statistical confidence that they are above or below the specified thresholds without the need to determine a quantitative value.

## Testing in Support of Labeling Finished Foods—Control and Compliance

To be certain that an analytical test method is appropriate for use with a particular food, the method must be validated with that food. Given the large number of different food substances and the extensive effort required to validate methods, it seems unlikely that large scale, routine testing of finished

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foods will be implemented. It is more likely that routine testing will focus on a limited number of agricultural commodities and processed food fractions that are at a point in the production process somewhere before the finished food. Threshold testing of incoming and intermediate processed fractions as part of a complete IP system and documentation of the use of these fractions in the preparation of finished foods allows producers to control their processes to provide high confidence that they are in compliance with food labeling laws.

While food producers will use testing to help them control their processes, regulators will use testing to determine whether producers are complying with labeling laws, and this may very well involve testing of finished foods. These are 2 different applications and may require different tests and strategies. The 2 technologies available to test biotech foods are immunoassay and PCR, and, of the 2, PCR may lend itself to the development of multi-analyte screening methods more readily than immunoassay. In addition, time and cost are not as critical in a regulatory setting as they may be in a production setting. It is yet to be seen whether PCR methods can be developed to quantitate the total concentration of biotech ingredients in complex finished foodstuffs. An approach for routine assessment of compliance may be to develop semiquantitative, multi-analyte PCR screening methods for final foods that would serve to alert regulators to a potential labeling problem. Once alerted, regulators could examine production lot records for evidence of control, including test results to determine whether further investigation is warranted. A complete investigation would likely require results from a number of different tests and analyses to reach a more definitive conclusion.

It seems unlikely that immunoassays will be developed for analyzing a large number of finished foods; however, they are being used successfully on a large scale to test grain and processed fractions of grain. Although efforts continue to develop PCR methods to analyze finished foods, it seems unlikely that such methods will be used for routine testing of grain. A considerable amount of effort has gone into validating immunoassay and PCR methods for specific applications. Given the potential of both technologies to be used in support of food labeling, it is important that future work focus not only on validating specific methods but on establishing correlations between the 2 technologies.

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