USDA-GIPSA/AEIC Workshop

Grain Biotechnology Detection Methods Validation Workshop

February 24-25, 2000
Kansas City, Missouri

P.L. Hunst, AEIC Secretary

The workshop convened on the morning of February 24 with a welcome introduction from Steve Tanner (USDA-GIPSA) to the workshop and a background introduction to GIPSA. The mission of GIPSA is to promote and protect the integrity of domestic and global marketing of US agriculture. GIPSA is harnessing technology to streamline the grain inspection process and provide repeatable, consistent testing. Steve reiterated that GIPSA's mission is to "facilitate the marketing of US grain". The goal of this workshop was to assemble worldwide experts to present and discuss analytical methods and their validation.

Chuck Mihaliak (AEIC President) gave a short introduction to the Analytical Environmental Immunochemical Consortium (AEIC). AEIC was formed in 1993 and consits of 20-25 organizations (agricultural chemical companies, immunochemical kit manufacturers, private and university laboratories). The focus of the organization has been on antiobdy-based detection methods. The AEIC has published method validation guidelines, provided educational and training opportunities for government agencies (EPA in particular) and has hosted workshops in the past.

Session I of the workshop was chaired by Don Kendall (GIPSA) and was entitled "Sampling, Validation and Standard Reference Materials".

Larry Freese (USDA-GIPSA statistician) presented "Obtaining a representative sample for testing purposes". The objective of a study is to measure some characteristic of interest on a lot, i.e., population, universe, etc. How could we measure this? The entire lot could be inspected or more reasonably, could inspect a sample of the lot. What is a sample? A sample is any subset of a lot. A random sample is a sample where every possible sample from a lot has an equal chance of being selected. Why are samples taken? The costs associated with sampling are much less costwise and timewise than inspecting the entire lot. The disadvantage of sampling is that a sample is not likely to contain the same amount of a characteristic as the lot. The source of measurement error in an experiment is contributed by sampling, sample preparation and the analytical method. Probability theory describes the distribution of estimates from random samples—a bell-shaped curve. For grain inspection, the common sized sample is 125g which is about 790 corn kernels. Systematic sampling is usually employed when sampling grain since it approximates a random sample and it is more easily implemented in many applications. For example, if there are 10,000 files in a cabinet and 50 of these files is to be sample for an audit, the sample rate is one out of every 200 files (10,000/50). To do systematic sampling, a random number between 1 and 200 is chosen. If the number is 138, the first file selected is #138. The next file selected would be the 200^{th} file after #138 which would be #338 and so on. Systematic sampling works well as long as the lot does not have a systematic pattern, therefore, every unit has an equal chance of being selected. The types of samplers used at GIPSA include: diverter types, probes, pelican and Ellis cup. These types of samplers yield a large sample that must be made smaller for use in laboratories. The large samples are further divided by using: Boerner dividers, cargo dividers or Garnet dividers. In summary, sampling is cost effective. Random sampling is desirable because probability theory can be used for analyzing outcomes. Systematic sampling is used to obtain samples that approximate random sampling.

Tom Whitaker (USDA-ARS) presented a talk on designing sampling plans to reduce the buyer's and seller's risks when inspecting grain from GM seed. It is difficult to measure the true attributes of a population based on samples. The true attributes can never be measured with 100% confidence because there is always uncertainty built in. This uncertainty results from sources of error, i.e., sampling error and analytical error. If the variability from these errors can be determined, then an Operating Characteristic (OC) curve can be drawn. Variability causes an OC curve not to be perfect. To reduce variability, one can increase sample size or increase the precision of the analytical method. If it is assumed that there is a 1%

tolerance for GM seed, a sample of 5000 seed would yield 28% error in detection of GM seed. If the sample size is increased to 20,000 seed, the error drops to 14%. However, this improvement in error reduction does not keep increasing with increasing sample size. At some point it becomes not cost effective to increase sample size. For OC curves, increasing the sample size from 1000 to 3000 seeds causes a steeper curve, i.e., reduces risks. However, increasing from 3000 to 6000 seeds causes an even steeper curve but does not result in a dramatic jump in risk reduction. If the GM seed tolerance level is changed to 0.5%, the OC curve is shifted such that there is much less risk for the buyer of the grain, however, the seller's risk are huge. In summary, to reduce error, the sample size may be increased or the precision of the analytical method may be improved. By shifting the tolerance level, the level of one risk can be improved at the expense of the other risk.

Chuck Mihaliak (Dow AgroSciences) presented "Synergistic utilization of testing technologies". The ideal GM test characteristics are: 1) complete in less than 10 minutes; 2) cost less than $5/test; 3) be sensitive, reliable, accurate and reproducible; 4) quantitative; 5) internationally validated and accepted; 6) detects all commercial GM events; 7) can discriminate between EU approved and non-approved; and 7) proper reference materials are available. There is no single method that can do all of this. There is no single method than can simultaneously measure all events in the same product. There are no DNA/protein tests available which can determine the presence of individual biotech products. There are gaps between user needs and available methods. Why? The needs were not anticipated. The analytical methods were developed for other reasons (product development, registration, quality control, etc.). The analytical methods were designed to find a target analyte—not designed to confirm the absence of an analyte. Neither PCR or ELISA is well-suited for both quantitative and multi-analyte detection. Regulations for GM products are still evolving, therefore, the needs for analytical methods are not clearly defined. The questions that are being asked are: How much, if any, of the grain in a truck, barge, etc. is GM? Is all the grain approved for import into a specific country? Available analtyical methods measure how much protein or DNA is present, however, this is an indirect measure and an indirect answer to the questions. An accurate measure of GM protein and/or DNA may not always ensure an accurate measure of the % GM, the sampling, the variation in protein levels in plants, variation among methods, variation in reference materials and extraction efficiency. Some of the industry organization involved in answering questions and validating methods include: USDA-GIPSA, AEIC, American Crop Protection Association (ACPA), International Life Sciences Institute (ILSI), Committee on European Norms (CEN), American Association of Cereal Chemists (AACC), International Seed Federation (FIS), Joint Research Council (JRC), American Standards Testing Methods (ASTM E-48 Biotech Committee). There are a lot of people talking but there is also a need to harmonize together and avoid having this same workshop 8-10 times. What can we do? Define the best methods for each application. Communicate among developers and users of diagnostic test. Promote independent confirmatory analyses for enforcement actions. Cooperate in establishing validation guidelines, sampling plans, etc. Educate and train on the proper application of diagnostic technologies. And, last, but not least, establish realistic expectations regarding the capabilities of DNA and protein tests.

Cindy Lipton (Zeneca) presented "The Principles of Validation—An Overview". For immunoassays, the basics of validation are: method reproducibility (intra- and interassay); sensitivity (LOD, standard curves with appropriate concentration levels, adequate sample size); specificity (measuring analyte—not interfering substances); ruggedness (will method work in other labs); false positives/false negatives. The same questions are applicable for PCR along with: how is quantitation performed; how good are the primers used; false positives (caused by artifacts, cross-contamination, contamination with amplicons); false negatives (degraded DNA, contamination). In summary, method should match testing objective. Validation of any method requires the demonstration of performance characteristics. When choosing a method, one must consider the particular characteristics and challenges of the techniques.

Heinz Schimmel (Joint Research Center, European Commission) presented "Grain Reference Materials". Dr. Schimmel gave an overview of JRC and IRMM (Institute for Reference Materials and Methods). The IRMM is part of the JRC and was founded in the 1950's to support nuclear energy. In the 1970's, IRMM started producing reference materials for other aspects other than nuclear energy. The IRMM is the European counterpart of the NIST in the US. It sells reference materials to customers all over the world for use in agriculture, environmental and nuclear methods. The JRC is one of 20 directorates within the European Commission. The EU has passed a 1% tolerance level for GM but what does this mean? What is

it based on? IRMM has produced reference materials for Roundup Ready soybeans, Bt176 maize and Bt11 maize. These materials have been tested via quantitative PCR and quantitative ELISA. The ELISA was consistent in evaluating the reference materials for the three products. PCR, on the other hand, was not. The inconsistency is believed to be a problem of the method. To understand what this problem may be, one has to understand the principles of producing reference materials. Optimal homogeneity of the reference material requires harsh production methods (grinding, mixing, drying). If homogeneity is not achieved, method validation based on intercomparisons will fail. Reference materials are typically produced as follows: 1) drying and heating of the material to remove enzyme activity; 2) mixing of accurate mass fractions in an aqueous slurry; 3) freeze drying; 4) mixing; 5) bottling for storage and shipment. Task was to determine which of these steps influenced the PCR results. The mixing using non-propeller types of mixers and mixing slurries at higher temperatures were found to influence the results. With PCR, if 2% GM seed present, 2% GM DNA is yielded, however, the accuracy is very poor (10%). This was felt to be due to differences between homozygous, heterozygous materials (different molar fractions of DNA); multiple copies of the gene; multiple transgenic and native gene sequences due to polyploid genome; and specific DNA degradation (35s degrades more slowly than Roundup Ready). The requirements for future reference material production are: 1) identification of transformation event; 2) raw material from the same clonal population; 3) characterization of homozygous and heterozygous; 4) purity check by determination of amount of non-GM material; and 5) need genetic background of parental lines. The hoped for outcomes of future productions are 1) less degraded DNA in reference materials; 2) supply intentionally degraded materials for comparison; 3) optimization of production techniques to protein measurements; 4) production of reference materials of other varieties or species and 5) production of representative food fraction reference materials.

Steve Evans (Dow AgroSciences) presented “Protein Reference Materials”. Protein reference materials are produced for immunochemistry methods. The immunochemical process is a continuum, thus, protein spans the needs in the process. Many parts of the process are internal to the developing organization or company. The external needs are demanded by regulatory agencies and governments. The nature of protein reference materials is dependent on the nature of the question asked. The production of protein reference materials does not follow a general procedure because the procedure is protein dependent. Microbial production is cost effective, however, bacterial production does not result in post-translational modifications which may be important in the plant. The characterization of a protein reference material includes: 1) use of classic tools of protein biochemistry for identifying and quantitfying known components, endotoxins, glycosylation and moisture; 2) immunoreactivity; 3) stability; 4) homogeneity; 5) batch-to-batch transitions; and 6) multi-lab performance. For biotech, the production of reference materials is not on production path because the objective is to make seeds (product), not protein. Scale-up of protein is not critical to the product path. Scale-up is of seeds by the plant breeders. The main customers of protein are the regulatory groups. In summary, reference proteins are critical to the internal and external needs. Production of these materials is complex and pure protein in high quantity is not on the typical plant development pathway. New plant technnologies will increase the demands on the production of reference materials.

Stacy Charlton (Novartis) presented “DNA Reference Materials”. The function of a reference material is for protocol optimization, positive control for experiments, LOD determination and construct a dose response curve for quantitation. The types of DNA reference materials include grain mixtures, purified plant DNA and plasmid DNA. Grain reference materials best mimic real life and spiked mixtures can be prepared. There may be difficulties in maintaining them, however. Plant DNA may be extracted from the grain. The quality and quantity of the DNA can be checked and evaluated, allowing for adjustment of concentration and division into aliquots. Genomic DNA is labor intensive to prepare but it is easy to store. Plasmid DNA can be replicated to high numbers in bacteria. It is easy to purify and may provide greater consistency. It can be a potent source of contamination if handled carelessly due to the high copy number/mass ratio. The number of reference materials needed for grain detection is influenced by the diagnostic approach: specific (EPSPS) vs. generic (35s). It is also influenced by the number of products on the market—14 corn events vs. 1 soybean event. PCR requires a collection of positive controls rather

Facilitated Discussion of Session I (Dave Grothaus-Pioneer)

Is there some means that purified reference materials could be made available to develop assays, i.e., made available from technology providers to diagnostic kit manufacturers?

Steve Evans: I don't know if this is possible.

Heinz Schimmel: The IRMM would be open to cooperations to do this.

What would be involved in order to assess OC curves for GM analysis?

Tom Whitaker: We need to know the policy decision. Once we know the policy, we can go in and measure analytical variation, etc. and determine the OC curve.

What kind of test we will use—qualitative or quantitative—for seed and grain going to Europe?

Stacy: The FIS is meeting tomorrow to talk about testing. The process will be worked out based on a 1% level.

Is it true that all ring tests have been directed to the qualitative?

Heinz Schimmel: For PCR, all the tests have been qualitative. For ELISA, they have been both qualitative and quantitative.

What do you think is the molecular basis for the differential degradation of the housekeeping genes vs. the GM genes? How would explain differential degradation within the same genome?

Heinz Schimmel: My molecular backgroung is not extensive but I will try to explain. The degradation in the soybean are presumptions made on preliminary data. We have seen clear evidence on gels of enzyme degradation and it is site specific and it is consistent data.

Stacy Charlton: Lectins occur in gene families and could cause variability in the PCR. If there was a radical difference in amplicon size between the lectin gene (housekeeping gene) and the GM gene, this could result in more hits of the lectin gene. There are differences across genomic DNA to nuclease digestion and this is documented by anyone who has made a DNA library.

Should we base our analysis of OC curves on number or weight?

Tom Whitaker: OC curves depend on the number of seeds ground—not weight.

Do three samples of 100 seeds each give the same answer as one analysis of 300 seeds?

Tom Whitaker: Yes, but it is much more complicated. If you average the three, then it is the same. If the three are taken independently, it is much more complicated.

Why send out powder reference materials? Should the grinding be done by the user?

Heinz Schimmel: We would have to send out a lot of kernels and each kernel would have to be characterized. There would be problems in each lab to attain homogeneity. Grinding is part of the reference material preparation and it is part of the process to achieve homogeneity.

DNA analysis is related to a population genetic study. Can an OC curve be applied?

Tom Whitaker: I really do not know the answer to this question. If you can get numbers, then an OC curve could be developed.

What are timelines for developing reference materials for regulatory agencies? How can we keep up?

Stacy Charlton: Not a problem for a couple of years due to the moratorium in Europe. It will be a matter of how quick the grain will be produced.

Steve Evans: It is a stage process. For protein, the demands are tougher.

Heinz Schimmel: It is a difficult question for grain reference materials. We need to know the specifications of raw materials. It will probably take 1-2 years but this depends on the cooperation of companies providing the grain. We will try to make reference materials to products before they are released in Europe.

Chuck Mihaliak: The timing of the product introduction and what reference materials are needed are factors. Protein reference materials cost about $10,000 per gram. FTO issues make it difficult for companies to give away reference materials.

Is anybody paying attention to reliability of primers coming from commercial sources?

Barry Martin (Monsanto): For the ring test, all primers will come from Genesis. There is nothing providers can do about primers coming from other sources.

Heinz Schimmel: In Europe, the source of the primer can be different but the primer sequence must be the same for ring tests. There is no system for testing the quality of the primers, however.

Kim Magin (Monsanto): In the EU, once primer sets are validated, they becom eligible for use in the methods.

There was an inconsistency between the Roundup Ready reference materials based on CT. If the CT values between lectins, does inconsistency still show up?

Heinz Schimmel: The difference in absolute DNA levels creates the inconsistencies. We will make our data available and are planning to publish the results very soon.

What is the feeling concerning mixing test methods for food products and seed samples? If we can analyze individual seeds, it seems most appropriate to analyze the seed and determine by proportion analysis how much GM present? If you grind, will you end up with the same value?

Larry Freese: Seed is done by weight. These are some of the policy issues that we must have decisions on. How do we report—by seed, weight, etc.?

Session II was hosted by Barbara Van Til (USDA-GIPSA) and was entitled "DNA Detection: Polymerase Chain Reaction (PCR) Technology".

Alice De Lisle (Aventis) presented "PCR: A General Overview". PCR amplifies a specific segment of the DNA. This amplification is based on the normal activity of DNA polymerase. PCR requires a DNA template (reverse complement of single-stranded DNA), dNTP bases and magnesium. The enzyme Taq DNA polymerase is also needed to synthesize the new DNA. A typical cycle in PCR consists of: 1) 1-2 minutes of denaturation of the DNA at 94C; 2) annealing of the primers to the denatured DNA for 1-2 minutes at 50-55C; and 3) Taq polymerase construction of the new DNA at 72C. Each cycle of PCR exponentially increases the specific DNA sequence. There is trait specific PCR and event specific PCR. A trait is a specific type of gene such as Cry9C (Bt), bar (glufosinate tolerance) and pat (glufosinate tolerance). An event results from the independent transformation of the same gene into different plant cells. The inserted gene is flanked by different segments of the plant DNA in each event. Trait specific PCR can recognize specific genes in the plant but it cannot distinguish specific events. Event specific PCR recognizes the border between the transgene and the plant DNA, i.e., the unique insertion site of the

transgene which is specific for each event. The limits of any type of PCR include: 1) relatively high cost; 2) requires technical expertise to perform; 3) requires sophisticated equipment; 4) sensitivity requirements for analysis; 5) prone to contamination.

Heinz Schimmel (JRC) presented "PCR: Methods Validation". PCR validation work was performed by another branch of JRC called the Institute for Health and Consumer Protection. There are several validation schemes (AOAC, Fitness for purpose, ISO 5725). These schemes are less applicable for qualitative tests. The required number of concentration levels of these schemes (5 levels) is not always available. For Europe, it is important to have European recognition of validation studies and this is why the JRC is involved. The first PCR method validated was a qualitative one for soy and maize raw products. There were 29 participants from 13 countries. Each received 16 unknown samples. Each lab had to have a protocol for analysis based on performance criteria (fixed primers; no specification of reagents). The results of the study were that 98% of the negative samples for soy were reported as negatives. For maize, 98% of the negatives were reported as negative and 84% of 0.1% GM were reported as positive. There was also a study for processed products in soy and maize. For this study, there were 23 participants from 13 countries and each received 30 samples. Only labs with GM detection experience were allowed to participate. Some of the processed products such as polenta, infant food and biscuits, contained both soy and maize. The results overall were: a) one lab had contamination problems, i.e., reported everything as positive; b) false negatives were much less pronounced; and c) half of the labs had acceptable performance. A study on quantitative PCR is currently in progress. The method is competitive PCR rather than real time PCR. Real time PCR requires expensive instrumentation and few of these are in Europe. In summary, validated DNA-based methods are validated and more will become available soon. EU Member States want line specific PCR methods. The JRC is very much interested in cooperations with US institutions in order to normalize methods and standards worldwide.

John Fagan (GeneticID) presented "PCR: Qualitative vs. Quantitative". The GM thresholds require quantitative methods of detection. In the EU, PCR is preferred method. The % GM can be calculated on the basis of: $\%\ GM = \frac{GM\ marker\ x\ 100}{species\ specific\ marker}$

The molecular based ratio can be converted into weight based ratio. At GeneticID, extraction is customized for each sample that is received. Heterozygousity is not as big a problem as it appears because the genetic makeup of the plants is controlled by the seed companies (uniform gene copy number in the field). There is inherent sensitivity in PCR, however, it must be in the right hands to achieve consistent data. Conventional PCR is semi-quantitative. Competitive PCR is quirky and does not give any better results than semi-quantitative. GeneticID uses real time approach using the iCycler system. In summary, both DNA and protein based detection are useful in GM analysis. One must use analytical methods which conform to the specifications of the customer. Performance criteria should structured—not standardized methods. If we operate to performance criteria, this allows to test as the technology evolves. Methods go out of date quickly.

Mike Russell (Central Hanse Analytical Laboratories) presented "PCR: Practical Applications". The question is what methods will allow our customers to ship and sell their products? Central Hanse uses ELISA, dipstick immunoassays or PCR depending on what the customer needs are. For PCR, initial screens are conducted at 50 cycle reactions. PCR can detect very short fragments of DNA (190bp) within processed foods. The lab screens for 35s and nos terminator. Qualitative PCR is done by the electrophoresis method and quantitative PCR is done by the TaqMan system. The QC/QA is very important. All the appropriate controls must be run—extraction blanks, PCR blanks, negative control, speciment reaction control. The building must also be set up appropriately to avoid contaimination. The sample preparation area must be physically separated from the analysis area. Sampling is the biggest uncontrolled issue. The sampling should define the degree of homogeneity of the product and the intended use of the product.

Facilitated Discussion (Dave Hondred-Pioneer)

What efforts are underway to standardized test methodologies?

Heinz Schimmel: We still have a long way to go. There are no formal requirements which would be considered acceptable. In Europe, it is a political process.

John Fagan: There is a movement towards accreditation for labs to ISO standards. The same discussion is occurring in Japan and the Pacific Rim. American move fast so maybe we can settle this quickly.

Alice De Lisle: We are working with FIS. There is no threshold set but there is an experimental target set. The group is in the process of setting up experiments.

What about stacked traits—are there methods to detect them?

John Fagan: Yes, because we can have trait specific probes and primer sets. We can characterize any given trait from the stack. It should be possible to set up a ratio. The question is really about distinguishing a mixture from a stacked trait. More importantly, if one of the traits is not approved or if the stack is not approved, we will need to detect this.

Heinz Schimmel: We did not screen for virus (CaMV). Since not all the antibodies did not find, there was a cross contaimination problem.

Can you distinguish between Bt corn and Bt sprayables since both are engineered?

John Fagan: Sprays are topical and the probability of them being on grain is minimal. Bt sprayables are broken down by UV light quickly.

Corn starch is ubiquitous in the environment. What steps are being taken to eliminate contamination from these sources?

Mike Russell: Most samples coming from the production line and are in plastic bottles.

How big an issue is heterozygousity in quantitative PCR for breeding?

Alice De Lisle: We do not use PCR in breeding.

John Fagan: Seed is produced from two varieties resulting in an average level of heterozygousity. Is this right?

Stacy Charlton: One of the parents is converted to the transgene so the hybrid is hemizygous. The grain is not uniform because of self or cross pollination. The transgene will segregate 1:2:1.

John Fagan: If we average the whole field, it will be the same.

Stacy Charlton: Hybrid grain is only 75% of the progenitor. If the reference material is hemizygous, this will cause an overestimate because 25% of the grain does not contain the transgene.

Using the assumption about the number of copies of a gene, that may hold true for a particular field but what about grain coming from large areas and put in the hold of a ship? Does it still hold?

John Fagan: If you take a large sample, gene copy number will be hemizygous.

Heinz Schimmel: It would appear as heterozygous material. For reference materials, the genetic makeup of the material must be specified for the proper use of the material. Overall, the mean of the reference material is a constant factor. We could calculate a correction factor.

No one has addressed the time issue for PCR analysis. Time is money. How long does it take in the labs?

Mike Russell: The reality is that we are a new lab. We are advertising a 12 hour, a 24 hour and a normal turn. We want to achieve 3-4 days. Currently, it takes a week to 10 days.

John Fagan: We feel our shortest turnaround is 18 hours. We also have 36 hours and 3 days. Working with our customers, we can time appropriately for business.

How many samples and cost/time?

John Fagan: More than 80 samples lengthens the time for analysis. The triple check system costs $385 per sample. Screening is less…there is a discount for volume.

Mike Russell: There are 12 samples/TaqMan run. Extraction is really the limiting step. Grinding takes just so much time to accomplish.

Please comment on TaqMan for processed materials. Do you believe these are accurate results for these materials?

Mike Russell: The amount of DNA will limit quantitation. We can achieve a positive signal but there is not enough DNA there to quantitate.

Angus Knight (Leatherhead): Quantitation is relative to the external standard curve. If there is any inhibition, this will affect the ability to perform quantitative analysis. You need to have an internal standard.

Mike Russell: You need to design your QA plan correctly.

John Fagan: The key is to quantitate one GM marker and a species marker. We have seen some differential inhibition with the housekeeping gene. We have been able to deal with inhibition in other ways. If it is substantial, the precision will decrease.

Amplicon length. How does this affect GM testing?

John Fagan: It is critical with processed food. We cannot work with less than 80-90bp. We can still detect quite reasonably at this length.

Barry Martin (Monsanto): Technology is driving standards and there is an inherent conflict because PCR measures allele frequency. We have to be careful of the message we take from the data, i.e., DNA in a barrel of corn oil. Was it in the oil or was it a contaiminant? This is similar to measuring mycotoxins—if DNA is present, we said we could not eat the food. We must be very careful about the messages being sent from ring tests, etc.

The precision in PCR is 10%. In corn with 8 different events, we need to detect 0.1%. Does the variation really add up to 20%?

John Fagan: Most of us quantitate with 35s and we also do event specific with semi-quantitation. The field is not there for quantifying each event. We are only measuring 35s and the copy number is different in different events.

Does the precision error multiply?

Larry Freese (USDA-GIPSA): There would not be additive errors. The error would go up but it would not be additive.

Teri Dunahay (USDA-FAS): We should stress how important PCR implementation should be done by the right labs. In the EU study, 10% of labs had false positives. These are trade disputes just waiting to happen and these can have financial ramifications.

Heinz Schimmel: We should not just dwell on the outcome of the validation study. In the study, we can only take the first results and the labs are not able to repeat. In reality, the number would be lower because the labs would repeat to confirm the result. QA schemes are very important in the labs.

Mike Russell: We need an independent measure of accuracy as well as precision among the labs.

Could you identify the top three priorities to address harmonization, repeatability and accuracy?

Alice De Lisle: The first priority would be a pre-PCR screen. DNA testing is expensive. We need to give industries a primary screen which not so expensive. Biotech companies do not have adequate methodologies designed to release to grain processors and food companies. PCR should be a back up or confirmatory method.

John Fagan: The needs are harmonization and standardization. There should be some system of criteria to sort out labs. I would move forward with trepidation now because of the expense and risk in trying labs. We need a system to validate labs.

Heniz Schimmel: It is difficult question for us. We are puzzled by our results so far. We have to define future steps in more detail this is why I cannot say at this time. Generally, we will focus on validation of methods for GM plants. The data will soon be released.

Mike Russell: We are driven to mandate an approach by the marketplace. How do we segregate labs than can perform against those that cannot?

Has there been any estimated cost to the EU consumer for the testing of GM food?

Heinz Schimmel: No one has done this yet. The methodology will dictate and until it is defined, we cannot estimate. It will be expensive and will probably not be politically acceptable. I cannot comment on the political aspects.

John Fagan: EU retailers have made a big thing about keeping prices the same for non-GM and GM. It has not yet impacted the consumer. Retailers do have flexibility in the marketplace.

How is the JRC funded?

Heinz Schimmel: The JRC is financed by the European Commission (80%). The other 20% of our funds come from outside, i.e., sales of reference materials, etc. All JRC is non profit.

The focus has been on LOD…lower, lower, lower. GM crops have been shown not to be harmful. People involved with making GM technology work are justified in asking why? Japan uses 5% and EU is 1%. What is reasonable?

John Fagan: Variability in translating PCR numbers into GM numbers can be done. We need to agree on method and this is a regulatory decision. The same thing could be done with immunoassays. It is just a matter of standardization.

DAY 2

Session III was entitled "Protein Detection: Immunoassay Technologies" and was chaired by Kimberly Magin (Monsanto).

Dean Layton (Envirologix) presented "ELISA: A General Overview". ELISAs, as well as other immunoassays, use antibodies as the key reagents. Antibodies are produced by specific white blood cells against foreign substances. Antibodies bind to specific antigens and possess high sensitivity and specificity. Immunoassays have been used for over 30 years in the medical fields. They are highly reliable and have flexible test formats. Immunoassays are ideal for use when the test analyte is known. They are also economical for screening applications. The markets for immunoassays include environmental, food, industrial, pharmaceutical, veterinary and water quality. The common formats for immunoassays are microwell plates, coated tubes, coated capillaries, lateral flow devices and magnetic particles. The two types of immunoassays most frequently used are competitive and double sandwich. In the future, immunoassays will be developed to analyze multiple traits simultaneously; procedures will be simplified and optimized; and the tests will produce results faster.

Tim Gutomson (Midwest Seeds) presented "ELISA Multi-Well Technology: Methods Validation". Midwest Seeds uses immunoassays for high throughput analysis (500 samples or more per day). The assays are used to analyze GM traits in corn, soybean, canola, cotton, rice sugar beets. The samples come from nurseries, parent seed operations, hybrid production, grain production and soybean products. Midwest Seeds also conducts herbicide bioassays and does perform some PCR. For ELISA testing, 90-360 seeds are sampled and sequential sampling and pooling is employed to solve the cost issues for the cutomer. Primarily for corn, testing is done for Cry1Ab and Cry9C. ELISA kits that are on the market are generally used unless no kit is available. Then an assay is developed from "scratch". The normal parameters for validation are considered: extraction, optimization, selection of threshold, use of controls in tests and sample tracking.

Jim Stave (Strategic Diagnostics Inc.) presented "Lateral Flow Strip Technology: Methods Validation". Lateral flow tests are used by agbiotech companies, seed companies, seed distributors, grain elevators and food ingredient manufacturers. Sampling and testing is easily carried out in the field. Leaf punches can be made by using small tubes and corresponding caps to make the punch. For seeds, a single seed may be crushed with pliers in between layers of waxed paper. Following the addition of buffer, the resulting powder can then be tested. Sensitivity of lateral flow strips is determined during the manufacturing QC by using the purified protein of interest to test. Generally, the sensitivity is ppb (ng/ml). Lateral flow strips allow threshold screening of bulk grain. Existing sampling methods and equipment for grain can be employed. There have been some comparison studies done between lateral flow strips and ELISA and between lateral flow strips, ELISA and PCR. ELISA and the strips have shown for very good correlation in studies conducted to date. The correlation of lateral flow strips, ELISA and PCR were done in a ring study coordinated by the JRC. PCR exhibited consistent qualitative detection at 0.5% GM soy. ELISA exhibited consistent quantitative detection at 0.3% GM soy. Sensitivity is not the issue for the type of tests—application is the issue. Lateral flow strips were also found to work well in studies where food fractions were tested, i.e., soybean protein isolate, defatted flour, full fat flour, etc.

Charles Hurburgh (Iowa State University) presented "Practical Applications of Biotechnology Identification". In the grain market, grain types are segmented. They are handled in bulk, large shipments and are priced by their specifications. There is usually a $0.05 to $0.50 per bushel added value for meeting specifications. Approximately 25% of the grain produced will be specialty grain, i.e., high oil corn, high protein corn, white corn, low sat soy and non-GM. Identity preservation is controlling the grain from the grower to the user. It is generally used in niche markets, requires special transport and may add more than $0.50 per bushel value. For testing companies must realize that the bulk of the grain arrives at the elevator in a 7 day period during harvest. Last season, grain was sorted for oil content using NIR which caused a 15second wait per truck. This created problems when 100 or more trucks waiting to unload. Therefore, any testing for GM is going to have to be extremely fast. The sources of possible mixing of GM and non-GM grain are the original seed for planting. Seed companies need to be below 1% tolerance on seed, otherwise, their seed will out of specifications before they can even sell it. On the farm, the planter box and

cross pollination of the plants during growing are potential routes of mixing. During harvest, combines, wagons and elevator handling systems. During the 1999 season, a survey of 8 elevators was conducted to see how they were handling segregation. All but one were doing pre-screening of farmers' fields. All had separate dumps for GM. Seven had 1% tolerance on corn (for GM) and also gave 40-50% premium share back to the producers. Six elevators were using ELISA and two were using PCR. For sampling, four were using the multiple probe and four were using the diverter. All elevators were taking >2000g samples but were grinding 50-250g for testing. Testing required 5-20 minutes with 2 reps/load and each had 2 or more people conducting the testing. The GM segregation issue will not be solved by harvest of 2000. Farmers will need to keep watch on the market, read their sales contracts carefully and know what their neighbors are doing (to avoid cross pollination contamination). In summary, it is difficult to apply chemical testing to the grain market. It is going to require much customer interaction between elevators and farmers. The first point of sale (elevator) is the highest risk location for out of tolerance grain. Customer preferences are creating product oriented grain market.

Facilitated Discussion—Dwight Denham (SDI), facilitator

Immunoassays differ in the detection of Bt proteins. How does this affect the results?

Jim Stave: True. May not be able to detect all with immunoassays. Plants differ in expression of proteins. Varieties also differ in expression. All of these factors affect the quantitative aspects of the immunoassay.

With 1% threshold, what should seed threshold be?

Tim Gutormson: We do a 400 seed test. Most of our results are negative.

Charles Hurburgh: In order to keep the error down, the seed tolerance must be 0.2% and below due to the error stacking in the seed production chain.

If 50% of soybean crop was Roundup Ready, where did all of grain go?

Charles Hurburgh: Most non-GM programs for soybeans are centered on the STS technology from DuPont

Jim Stave: STS soybeans were found to be contaminated with Roundup Ready soybeans—1 Roundup Ready bean/3000 STS beans.

Tim Gutormson: Companies want some sort of documentation for contamination. We have usually seen 0.3-0.6%.

A question for the kit manufacturers—what is pipeline of products?

Dean Layton: We have kits for all events currently in our development pipeline. We hope to have some of these on the market by harvest time this year.

Jim Stave: We want to produce tests for all the events and we are advancing quickly in development.

Is cross reactivity a problem?

Jim Stave: There is no cross-reactivity between Cry1Ab and Cry9C. There is some cross-reactivity between Cry1Ab and Cry1Ac. For these, we may want to select antibodies with more specificity to each antigen to avoid this.

Does the protein content in plants change from season to season and/or in different geographical areas? When are there going to be kits for foods?

Dean Layton: Expression does vary between seasons and varieties. Processing affects the measurement capability of the proteins because the target proteins may be denatured.

Jim Stave: The Roundup Ready kit works with food fractions. SDI tried to work with food processors, however, the food processors did not want the tests. They want to push the testing and cost back to their suppliers.

Charles Hurburgh: The cost for testing food is going to huge because of the large number of products.

Is there liability in seed testing?

Tim Gutormson: We have insurance. The thresholds are not defined so it is difficult to do testing right now. We have a disclaimer but this does not fully protect.

What will happen this year?

Charles Hurburgh: Firms expect to handle more non-GM grain.

What work is being done to detect mutant EPSPS?

Jim Stave: It is difficult to make a test which would distinguish. The event may not continue in production so then it becomes a matter of economics for the kit manufacturer.

How will multiple traits be dealt with?

Dean Layton: We will start with individual event tests and then graduate to multiple events on strips. There are many technical questions that have to be answered before these tests are ready.

Jim Stave: It is a matter of practical application. There are 1 or 2 significant events in production. Immunoassays will pick these up due to their prevalence. Do you really need tests for every event or just the significant ones?

What is the feasibility of DNA detection dipsticks?

Dean Layton: There are a number of new technologies in the medical field for DNA. There will probably be none of these available in the agriculture field for the next 1-2 years.

Jim Stave: There are chip technologies and others out there but the existing technologies have to be validated. These new technologies do not solve the testing the problems for the grain that is currently in the bins.

In the studies presented, there was an unacceptable range of results for non-GM grain. Is this the rule or the exception? If the tolerance had been 5-8%, all the grain would have been classified as non-GM.

Jim Stave: We have to establish standard methods and correlate. The purpose of showing the data was to try to show correlation of the techniques. The methods should agree at least on the qualitative level. These are currently not acceptable for trade commodities.

Dean Layton: We need to have reliable reference materials in order to standardize methods.

Can a producer afford not to test?

Charles Hurburgh: Producers are confused for 2000. They will continue as they have in the past if the local markets are not yet affected. Livestock feed producers will not change.

IP systems will develop to control the chains of grain. What premium does a grower need for a non-GM program?

Charles Hurburgh: IP is expensive also. If there is no yield differential, $0.20 to $0.40 per bushel added value will cause a change in production. If there is a yield differential between GM and non-GM, it is harder to cause a change.

Jim Stave: Does a farmer test or go to IP? Both cost money. However, the clock is ticking for PCR or immunoassay testing, i.e., the methods must be validated and reliable. The IP solution is already out there and the farmer can see it.

What are the three top priorities to work on?

Dean Layton: Produce kits for key commercial events and optimize adequately. Also, there is a need for multi-analyte detection systems.

Jim Stave: Harmonize and the testing problems could be solved quickly. Some sort of IP systems are inevitable.

Charles Hurburgh: We need system analysis for efficiency. We also need sampling procedures and multi-analyte tests.

Tim Gutormson: It all comes down to cost. IP will probably be the way to go. Accreditation of labs must happen quickly.