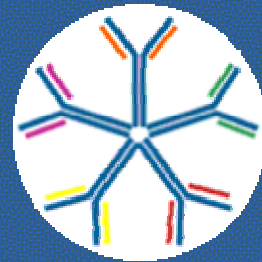


Methods for Detecting and Measuring Ag Biotech Products



AEIC
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Copyrights and Acknowledgements

AEIC is pleased to provide the following slide presentation for use in educational or training applications associated with detection methods for biotech products. Due to the size of the file, this presentation is provided as a PDF, which does not allow for any changes in content. For a copy of the presentation on a CD please contact AEIC.

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- SDI
- USFDA



Ag Biotech Crops

- **Transgenic plants have:**
 - Novel trait (e.g., herbicide resistance)
 - May express novel protein
 - Novel DNA
- **Novel DNA and protein may be found in:**
 - Plant tissues
 - Seed/Grain
 - Food ingredients and food products



Biotech Crops 2004

- **2 major traits**

- Insect protection - *Bacillus thuringiensis* (Bt)

- Herbicide tolerance

- Roundup Ready (RUR)

- Liberty Link (LL)

- Bromoxynil tolerance (BXN)

- **4 major crops**

- Corn - Bt, RUR, LL

- Soy - RUR

- Canola - RUR, LL

- Cotton - Bt, RUR, BXN



Testing in Support of Labeling Biotech Foods

- **Consumer Choice**
- **Approved Events – Quantitative and threshold testing**
 - **European Food Labeling Law**
Labeling began April 10, 2000 and updated April 2004
Threshold adventitious < 0.9% - "genetically modified"
 - **Japanese Food Labeling Law**
Labeling began April, 2001
Threshold guidelines set at 5%
- **Unapproved Events - Detection**
 - **Zero tolerance** e.g. StarLink
 - **European Food Labeling Law**
Threshold adventitious < 0.5% - "genetically modified"



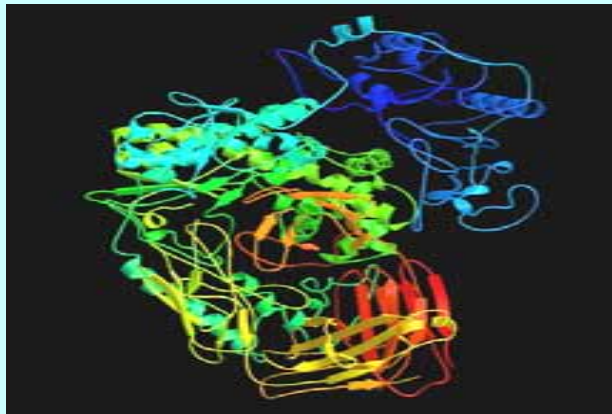
Determining Concentration of Biotech Ingredients in Foods

- **Results are reported in terms of % Ag Biotech**
e.g. 1 Biotech corn kernel in 99 negative = 1%
- **Decisions are based on regulated thresholds**
(given in weight %)
- **Testing is based on detection/quantitation of novel DNA or protein**
- **Ag Biotech concentrations are estimated from protein concentration**
- **DNA can be measured in relative terms, i.e. % Roundup[®] Ready soybeans with respect to total soybean**

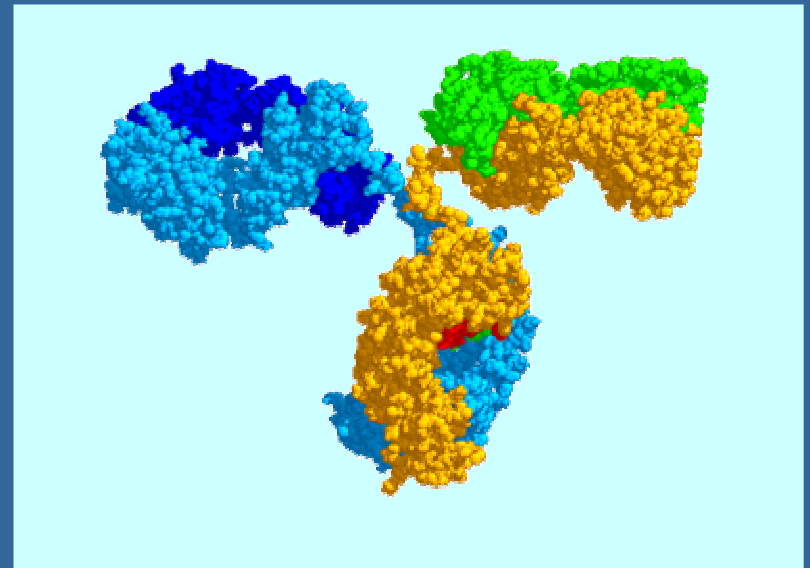


Commonly Used Detection Methods

DNA-based methods
PCR



Protein-based methods
Immunoassay (ELISA)



Biotech Immunoassay Method Validations

- **Collaborative studies**

 - AACC

 - MON810 Cry1Ab ELISA – ground corn

 - StarLink Cry9c ELISA – corn flour and meal

 - Joint Research Centre, European Union

 - Roundup Ready[®] ELISA

 - IRMM ground soybean certified reference materials

 - Soy toasted meal, protein isolate, defatted flakes

 - FDA

 - StarLink Cry9c ELISA – processed food fractions

- **USDA Certification**

 - Cry9c strip tests – corn kernels

 - Cry9c ELISA - ground corn, meal, flour

 - CP4EPSPS Strip tests - ground corn, soybeans



Immunoassay Performance Characteristics

- **Sensitivity (LOD, LOQ) - ppb to ppt (10^{-12} M)**
LOD - level of detection LOQ - level of quantification
- **Specificity**
 - Families of chemicals vs. single compounds
 - Commercial products
 - Metabolites, degradation products
 - Process by-products, intermediates
- **Precision - repeatability, reproducibility**
- **Accuracy - recovery and false negative/positive rates**
- **Matrix effects/interfering substances**
- **Quantitative range**
- **Stability, Reliability, Robustness**
- **Fitness for purpose**



Sample Extraction

- **Matrix effects**
 - Sample matrix - Extraction of the protein also extracts other substances from the sample
 - Interfering substances - Substances affecting assay performance
- **Extraction efficiency**
 - The % of target protein extracted by the method protocol
 - Does not need to be 100% - must be consistent
 - Balance
 - Sensitivity
 - Cost
 - Time
 - Ease-of-use

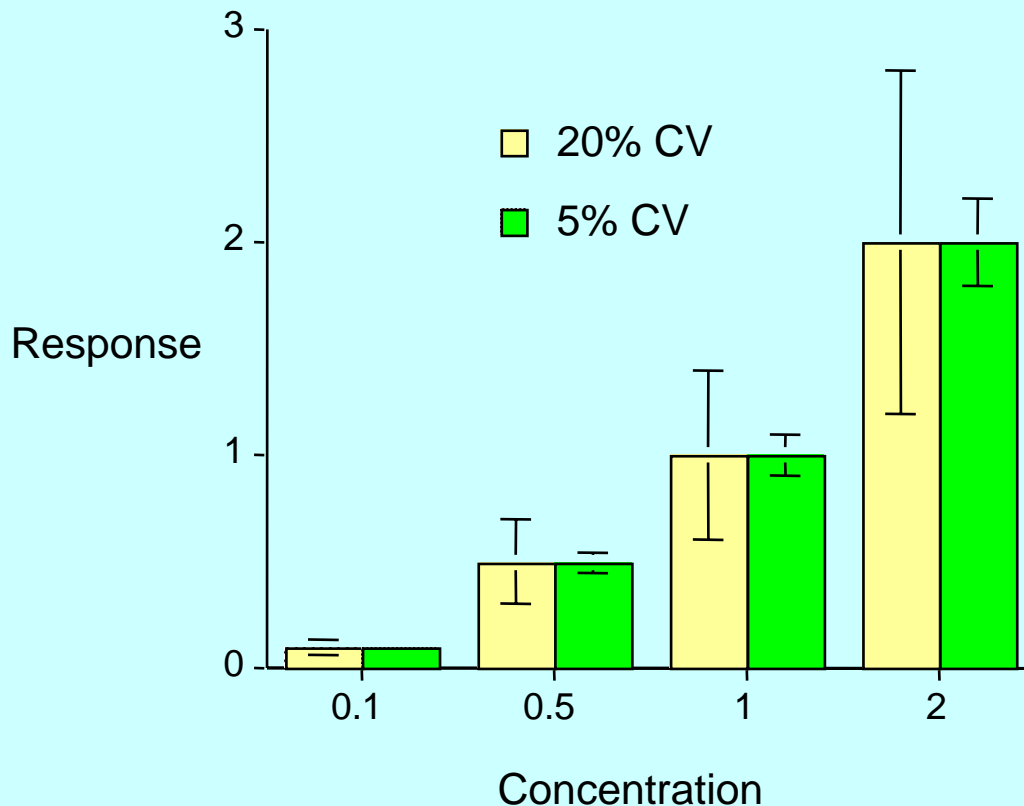


Precision

- The closeness of agreement between independent test results obtained under stipulated conditions
- The amount of variability due to random error
- Expressed as the standard deviation (sd) or coefficient of variation (cv)
- Determined by testing replicates of samples
- The precision of the final concentration must include variability of sample preparation
- Good precision provides ability to distinguish closely related concentrations
- Repeatability (reproducibility) is precision under repeatability (reproducibility) conditions
 - Repeatability conditions, test results obtained in the same lab, same method, same conditions, same operator, within short period of time
 - Reproducibility conditions, test results with the same method in different labs, different operators and different equipment



Effect of Precision on Ability to Discriminate Between Concentrations



Error bars for concentrations determined by the method having a 20% CV overlap. Both methods give the same concentration but it is not possible to say that adjacent concentrations differ from each other in the assay with the 20% CV within the given confidence interval.



Accuracy

- **The closeness of agreement between the (or a) reported result and the accepted reference value**
- **Quantitative methods**
Expressed as '% Recovery' of 'true' value
- **Qualitative methods**
Expressed as rate of false positive and negative results
- **Influenced by**
 - Precision
 - Bias
 - Matrix effects



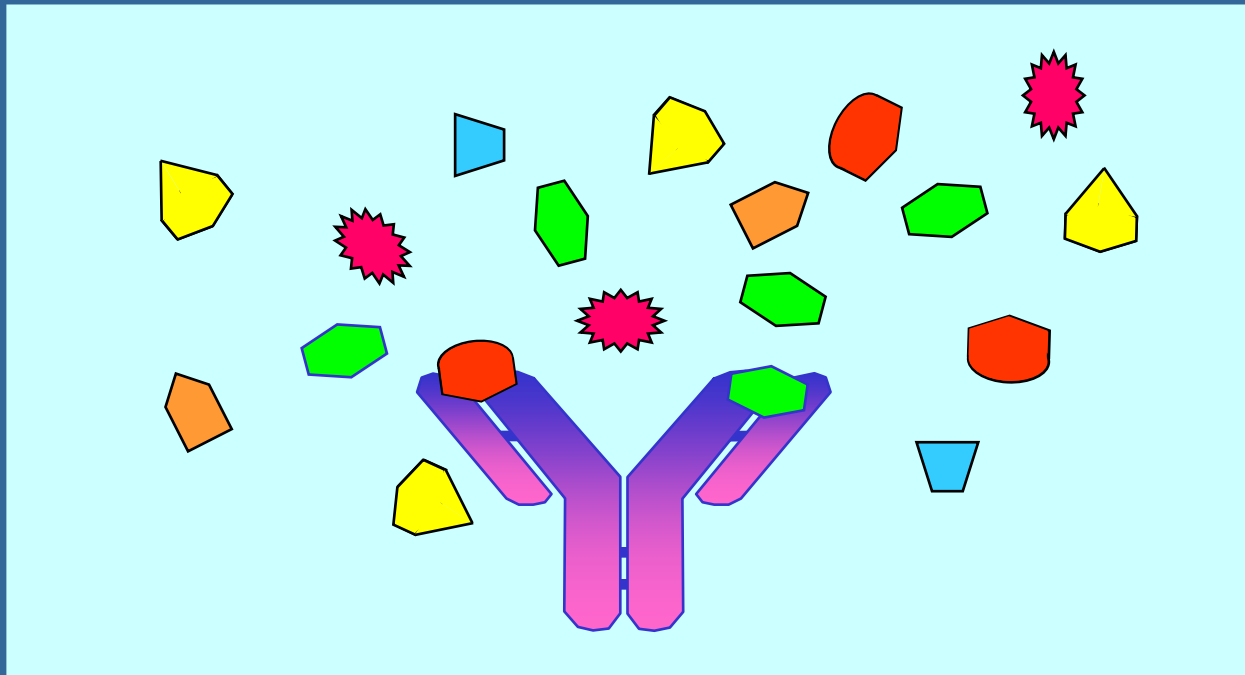
Sensitivity

- **Change in the response divided by the corresponding change in the concentration of a standard (calibration) curve; i.e. the slope of the analytical calibration curve**
- **The minimum concentration of the target analyte (e.g. protein) that can be detected or quantified**
 - Limit of detection (LOD) – The concentration of protein which can just be detected by the assay (e.g. 50% of the time)
 - Limit of quantification (LOQ) – The concentration of protein that can be quantified with stated precision (e.g. $\pm 10\%$)
- **Total method sensitivity must account for extraction efficiency**

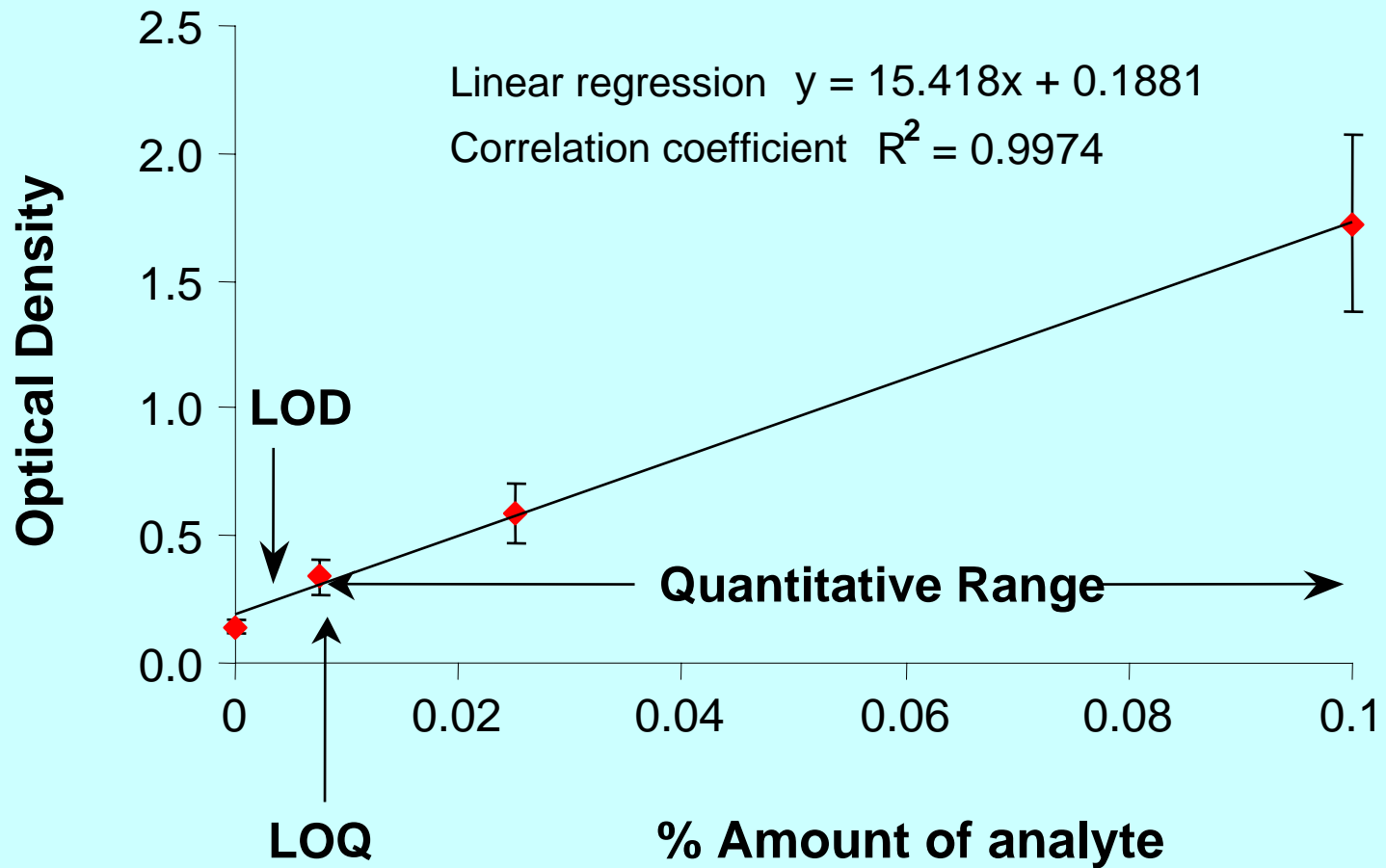


Specificity / Crossreactivity

- **Specificity:** the property of a method to respond exclusively to the characteristic or analyte (e.g. protein) defined in the method
- **Crossreactivity:** antibody binding at the binding site with substances other than the analytes of interest



Standard Curve



Standards and Controls

- **Quantitative Methods**

- A series of standards (calibrators) are used to construct a standard curve
 - Matrix-matched standards (e.g. corn flour)
 - Samples and standards are normalized for matrix effects and extraction efficiency
 - Must create standards for every matrix
 - Purified protein standards
 - Must establish that they give same response as protein in matrix

- **Qualitative methods**

- Negative control
- Positive control e.g. threshold concentration, need to determine LOD
- May have multiple levels to group results in limited ranges (buckets)



Factors Effecting Use of Protein Immunoassays

- **No protein – no Immunoassay**
- **Very low level expression (e.g. Bt 176)**
- **Crossreactivity (e.g. GA21 Roundup[®] Ready corn)**
 - Modified corn EPSPS – 2 amino acids of 445 different from native corn EPSPS

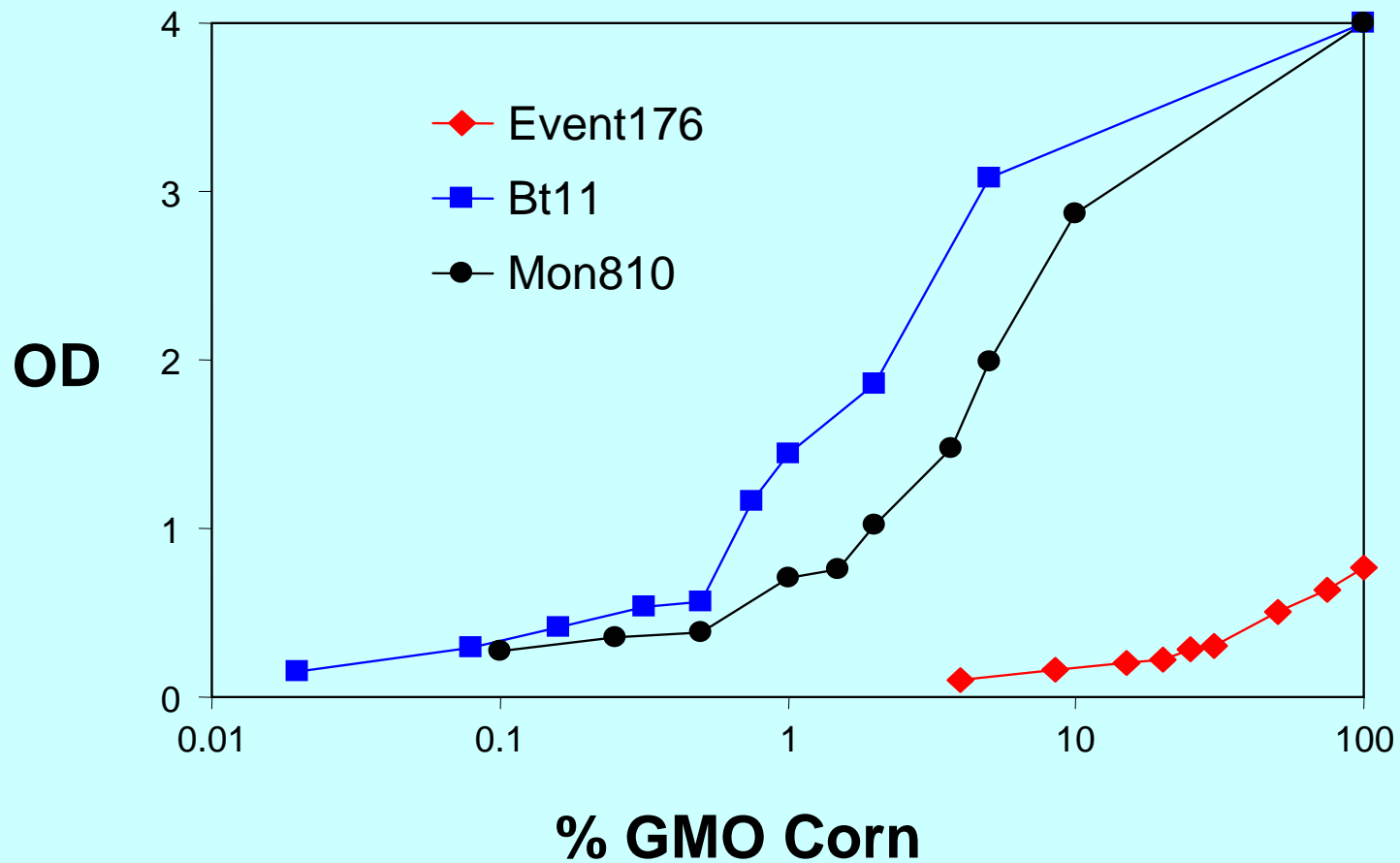


Factors Effecting Quantitation Using Immunoassays

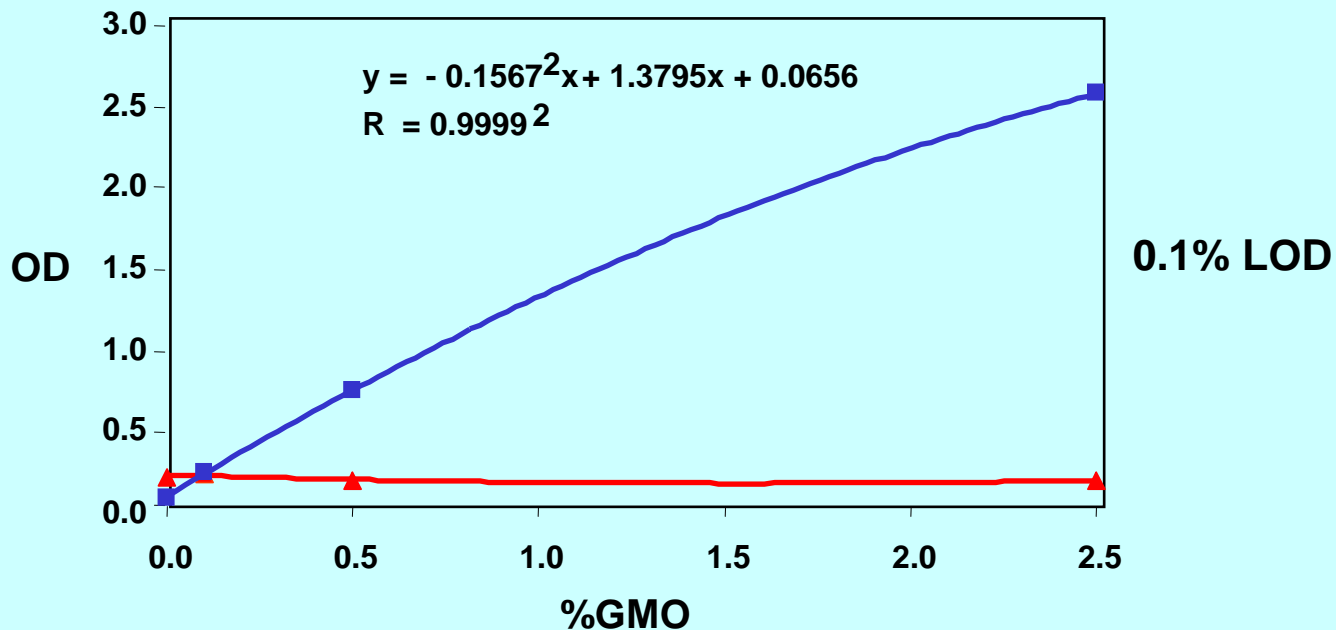
- **Method performance characteristics (e.g. precision, accuracy)**
- **Variability of protein expression levels**
 - Within an event
 - Between events expressing same protein (e.g. Cry1Ab)
- **Varied effects of sample processing on protein conformation and antibody binding**



Reactivities of Different Varieties of Bt Corn in Cry1Ab ELISA



Reactivity of 2 Different ELISA to RUR Toasted Soy Meal



- Antibody detects toasted soymeal
- Antibody not selective for toasted soymeal

Soymeal was toasted for 60 min. at 100 °C



Assay Validation/Bridging for Key Tissues

- Precision → inter-plate, intra-plate, inter-assay variability
- Accuracy → extraction efficiency, matrix effects, spike and recovery, robustness, ruggedness
- Sensitivity → limit of detection, limit of quantitation
- Specificity → matrix effects, specific for trait



False Positive Results

Problem	Control Measures
<p>Artifact PCR products from templates other than the target sequence in question (worst scenario: artifacts of the same size as the expected PCR product)</p>	<p>During validation:</p> <ul style="list-style-type: none">▪ Search data base for potential unwanted (homologous) annealing sites▪ Test new primer system on a wide range of DNA types that appear in food samples▪ Verify the identity of the PCR product by Southern blot or restriction enzyme digestion
<p>Corruption of sample, extracted sample DNA or PCR setup contains positive DNA</p>	<p>During routine analysis:</p> <ul style="list-style-type: none">▪ Follow strict rules how to work cleanly in a molecular biology lab▪ Use one way sample flow lab design to prevent contamination with positive DNA from downstream steps (especially PCR products), strict separation of certain lab areas from each other▪ Use duplicate analysis of each sample▪ Use negative controls on homogenization, DNA extraction and PCR setup



False Negative Results

Problem	Control Measures
Insufficient sensitivity of primer system	During design and validation: <ul style="list-style-type: none">▪ Use computer aided primer design▪ Test the sensitivity of new primer systems for the intended concentration range
Inhibition of PCR reaction	During validation: <ul style="list-style-type: none">▪ Test primer system on sample DNA solutions known to contain inhibitory compounds During routine analysis: <ul style="list-style-type: none">▪ Test each PCR reaction for inhibition by an individual positive control (a spiked counterpart)▪ Use duplicate analysis of each sample
DNA extraction failed	During design and validation: <ul style="list-style-type: none">▪ Choose appropriate extraction protocol for sample type▪ Run controls to monitor efficiency of each extraction series

