

Two Case Studies: Verifying Methods to Detect Inadvertently Released Grains into the Marketplace

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Overview

- **Introduction to Method Validation**
- **Evidence of reliable detection of Bt10**
- **Evidence of reliable detection of LL601 with 35SBAR PCR method**

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Method Validation

- Verification that an analytical method is acceptable for its intended purpose
- An essential component and one of the measures universally recognized as a necessary part of a comprehensive system of quality assurance in analytical chemistry and food technology
- Standard used to demonstrate compliance of food labeling laws
- Specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness

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Validation of a Quantitative PCR Method

- **Method Performance**
 - Determines bias and precision of the analytical method
- **Material Performance**
 - Assigns a value and an uncertainty (or reliability) to a characteristic (usually concentration) of a material
- **Laboratory Performance**
 - Permits the evaluation of each participant against preset criteria

Lipp et. al. J AOAC Int. 2005

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Acceptance Criteria

- **Positive Target DNA Control (contains target)**
demonstrates that test samples perform as predicted
- **Amplification Reagent Control (does not contain target)**
contains all reagents except test sample, template DNA
- **Relative Standard Deviation (RSD) less than 33% RSD**
at all target concentrations within the dynamic range
(JRC and Horwitz)
- **Trueness**
+/- 25% throughout the whole dynamic range of fortified
samples (European Network of GMO Laboratories)

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What is the Limit of Detection (LOD)?

The lowest quantity of a substance that can be measured as greater than “0” with a high level of confidence

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Biotech Plants on the Market

- **Agronomic Traits**
 - **Herbicide Tolerance**
Roundup Ready soybeans, corn, canola, cotton
Liberty Link corn, rice, canola
 - **Insect resistance**
 - Bt corn
 - Cry 1AB (Mon 810, Bt 11, Bt 176)
 - Cry 9C (Starlink)
 - Cry 1F (Herculex)
 - Cry 3Bb1 (Mon863)
 - Cry3a (MIR604)
 - Bt cotton (Bollgard)
 - **Stacked Traits**
Bt11xT25, Mon810xNK603, DAS59122xTC1507xNK603, others

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Legislative Mandates and Benefit vs. Risk of Biotechnology Derived Events

- **United States**
 - Substantial Equivalence**
 - Toxicity
 - Nutritional Content
 - Allergenicity
- **Europe**
 - Consumer Choice** (Perceived Risk)
 - Labeling required if threshold above 0.9%

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Grain Market Needs

- To distinguish biotech from conventional crops, the market needs testing techniques that are:
 - accurate
 - reliable
 - cost-effective
 - market compatible
- Testing can be combined synergistically with efficient and reliable IP (Identity Preservation) systems

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DNA-Based Testing Issues: Quantitation

35S and NOS

- Multiple copies
- Units: % weight, % DNA material
- No Internationally recognized reference materials (ie. 35S)

Event Specific

- Multiple copies
- Units: % weight, % DNA material
- Cannot distinguish between single events and stacked events
- Technology limitations (PCR)

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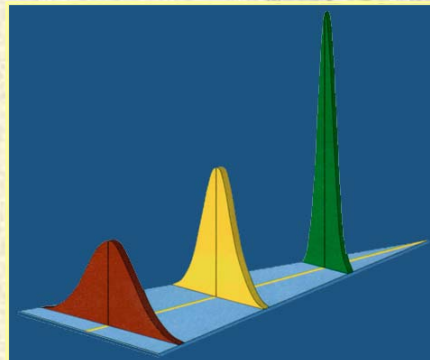
GIPSA/FGIS' Mission

**“Facilitate the marketing of
U.S. grain
and assist in resolving
disputes between
buyers and sellers”**

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USDA's Role

**USDA can facilitate
the marketing of
Biotech and non-
Biotech grains by
improving and
standardizing the
detection methods
for Biotech grains.**



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**Why use PCR rather than
ELISA or Lateral Flow tests?**

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ELISA LOD: 1.33%
(1 kernel in 75)



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PCR LOD: 0.01%
(1 kernel in 10,000)



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Polymerase Chain Reaction (PCR)

- **PCR allows for rapid amplification of specific target segments of DNA through mimicking in vivo replication**
- **Multiple cycles of DNA synthesis may produce a million-fold increase in the target DNA**
- **Grains not containing these DNA fragments will not produce a response**

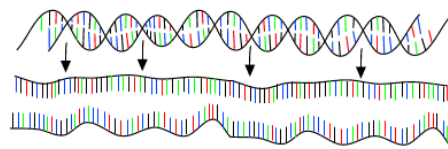
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How does PCR really work?

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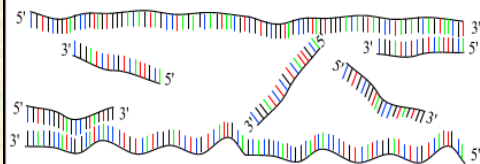
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

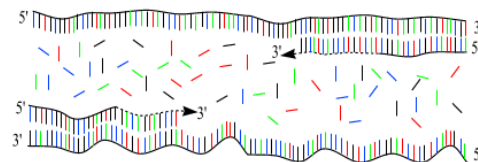
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



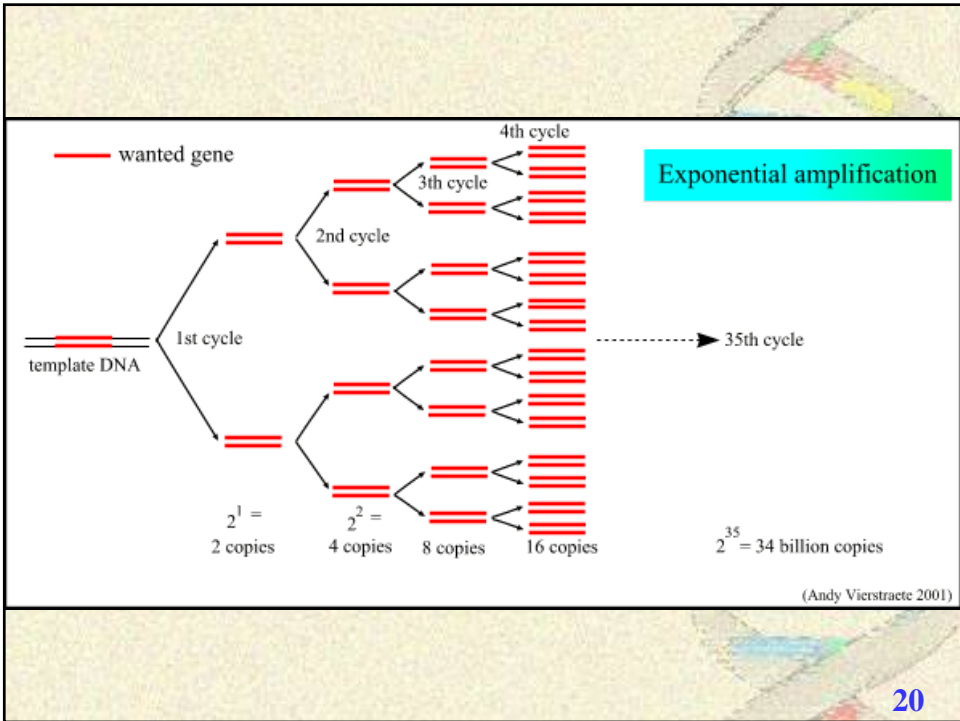
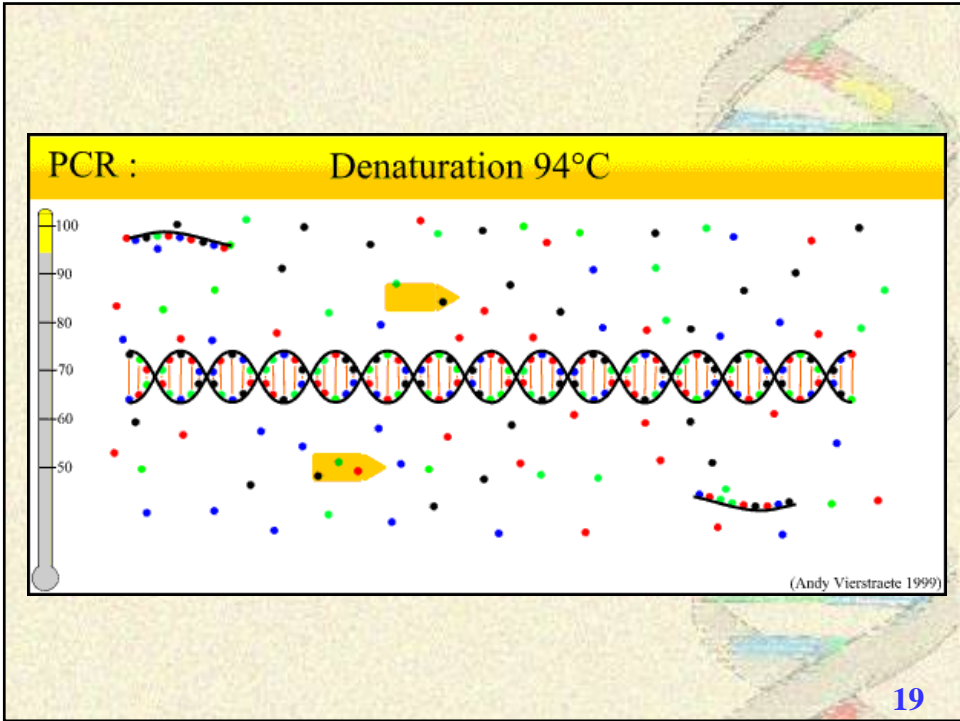
Step 3 : extension

2 minutes 72 °C

only dNTP's

(Andy Vierstraete 1999)

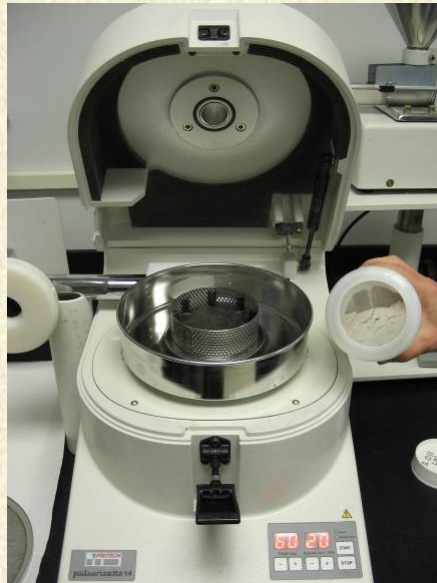
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How are PCR tests performed?

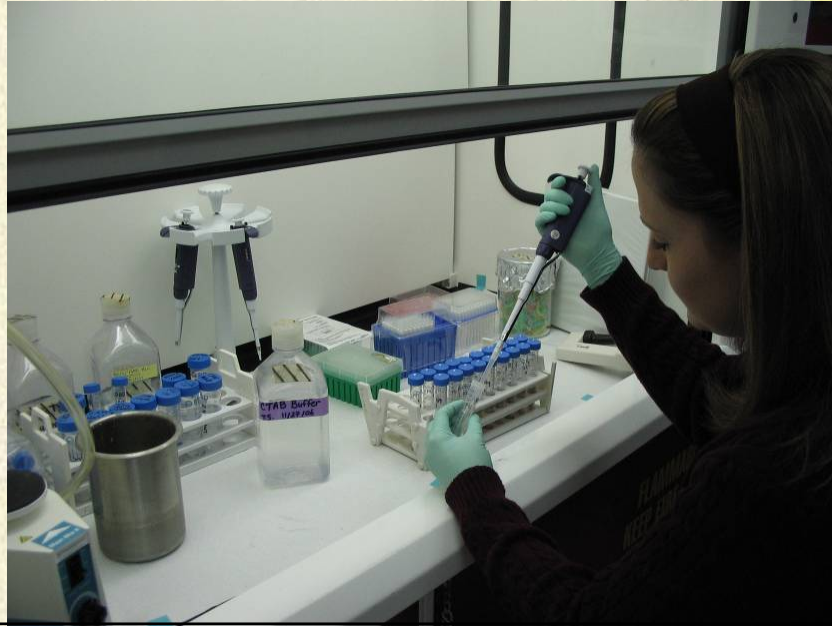
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Grind to fine flour under clean-room conditions



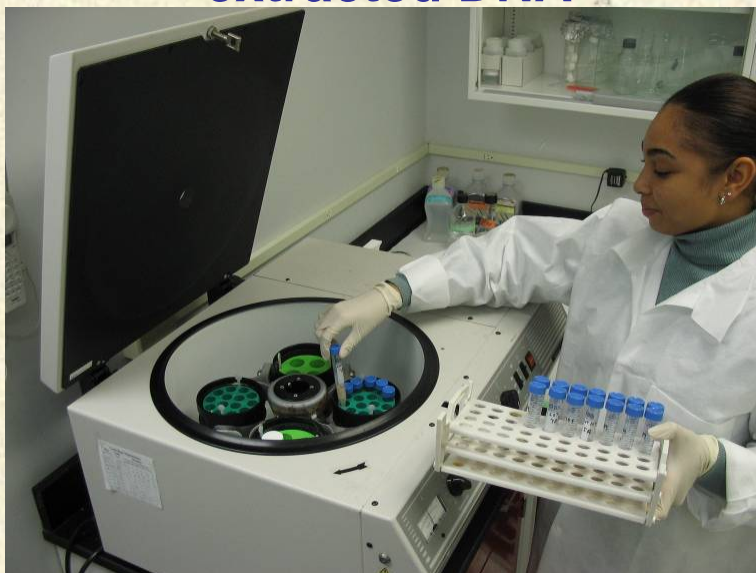
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Add extraction solution



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Centrifuge to separate extracted DNA



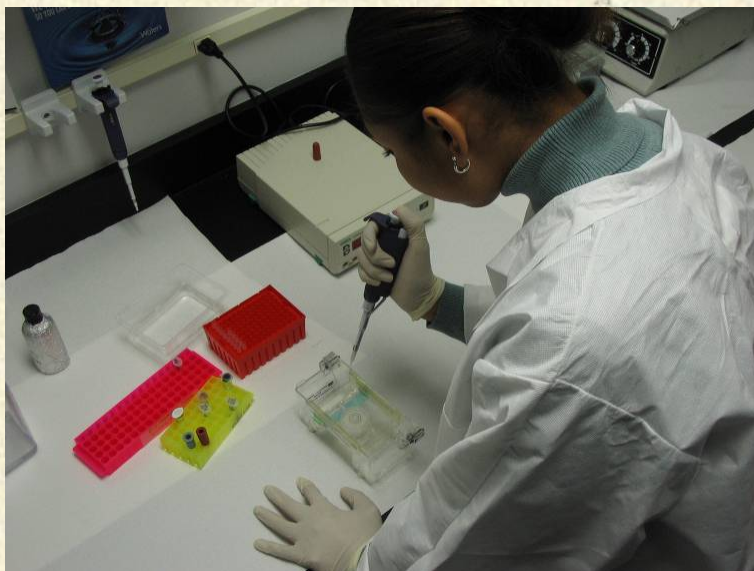
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Quantify extracted DNA by fluorometer



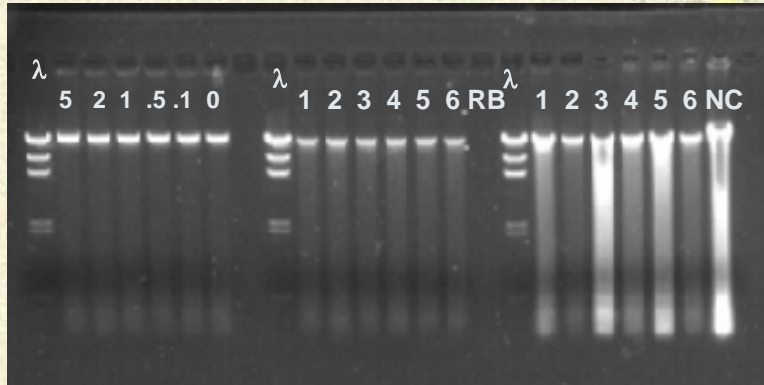
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Verify DNA purity/integrity by electrophoresis



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0.8% Agarose Gel Electrophoresis (100 ng Genomic DNA/lane)



✓ Accept

✓ Accept

X Reject

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Pipette DNA into 96-well PCR plate



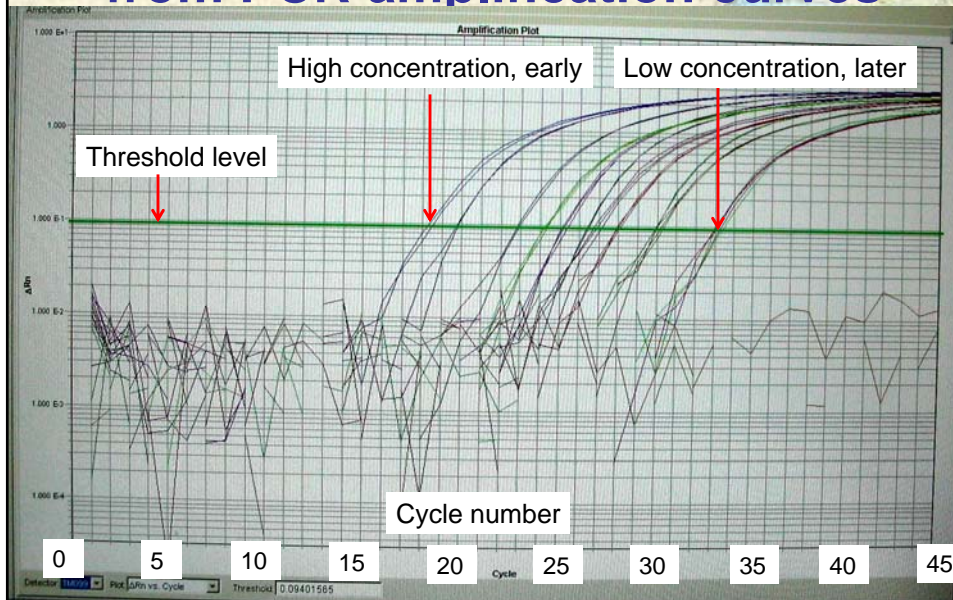
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Place 96-well plate into real-time PCR instrument

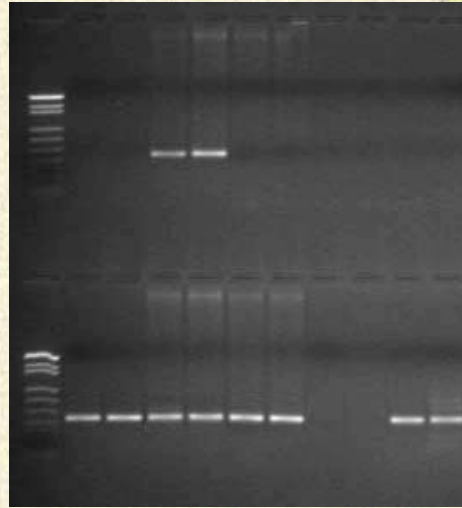


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For Real-Time PCR read results from PCR amplification curves

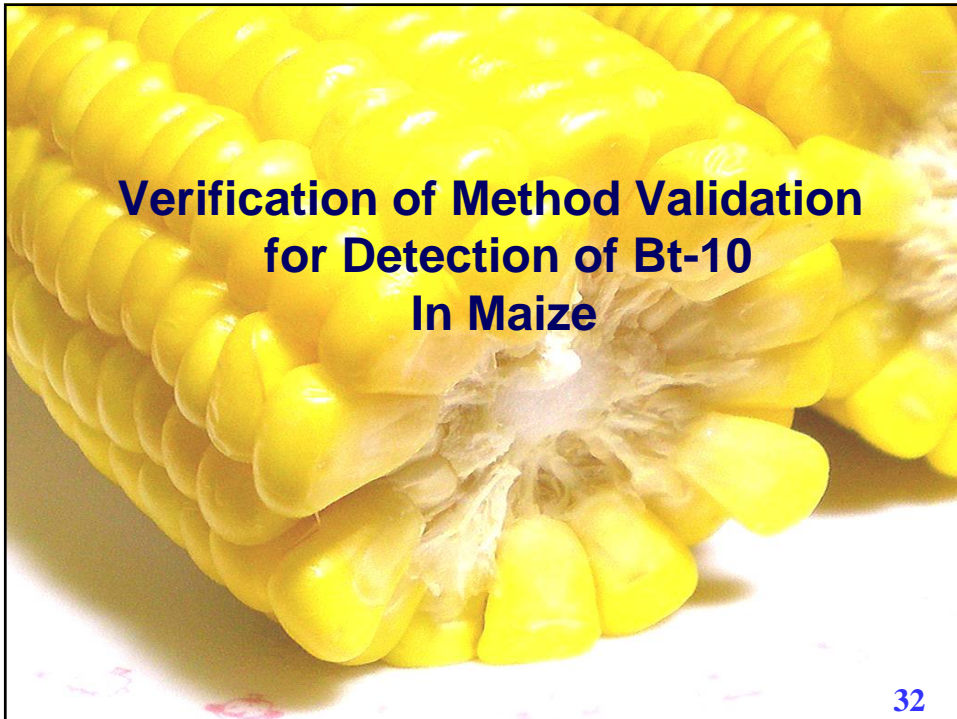


**For Conventional PCR
Read results using agarose gel
electrophoresis**



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**Verification of Method Validation
for Detection of Bt-10
In Maize**



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Background on Bt-10

- A Syngenta product, inadvertently mislabeled and low levels were planted and sold as Bt-11 between 2003 to 2005
- Contains the Cry1AB protein (Identical to Bt11)
- Not a government approved event
- Contains an inactive ampicillin resistance gene not found in Bt-11

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Legal vs. Health Issues

- The incident disrupted trade of corn gluten feed and related products
- Incident was reported by Syngenta to the USDA
- Bt-10 poses no human health risks

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Bt-10 Method Validation

- **GeneScan**
 approached by Syngenta to develop and validate a method for detection of Bt-10
 gene sequence made available by Syngenta
 (Confidential)
- **GIPSA Role**
 Independent verification of the GeneScan method

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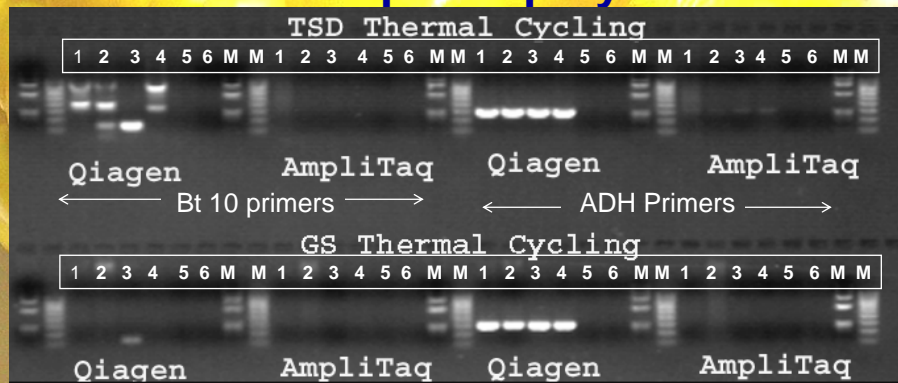
TSD Thermal Cycling (Qiagen Reagents)

The gel image displays two rows of bands. The top row is labeled 'Bt10 Primers' and shows bands for '0.1% Bt10', '0.0% Bt10', and 'spiked' samples. The bottom row is labeled 'ADH Primers' and shows bands for '0.1% Bt10' and '0.0% Bt10' samples. Arrows indicate the primer regions for each row.

*Note: Sigma Primers in 0.5 x TE

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Comparison of Two Different Thermal Cycling Parameters and Two Different Taq DNA polymerases

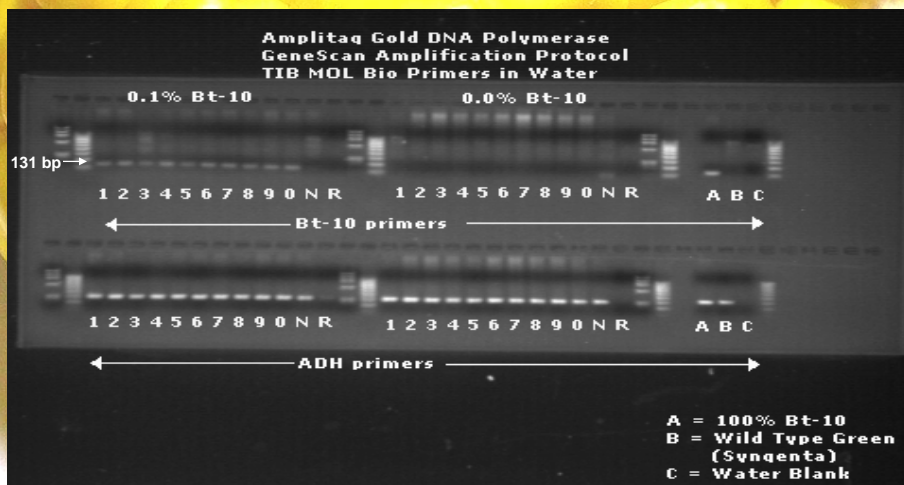


Lane **Lane** *Note: Sigma Primers in 0.5 x TE

1	0.0% Bt-10	4	wt green (negative control)
2	0.1% Bt-10	5	reagent blank
3	100% Bt-10	6	water blank

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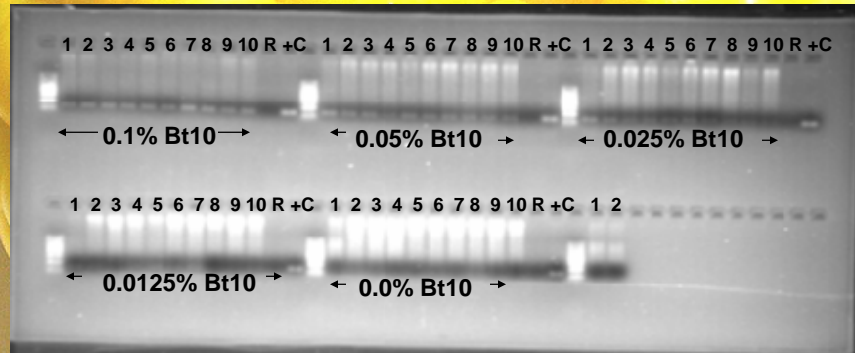
AmpliTaq Gold with GeneScan Thermal Cycling



*Note: Tib Mol Bio Primers in water

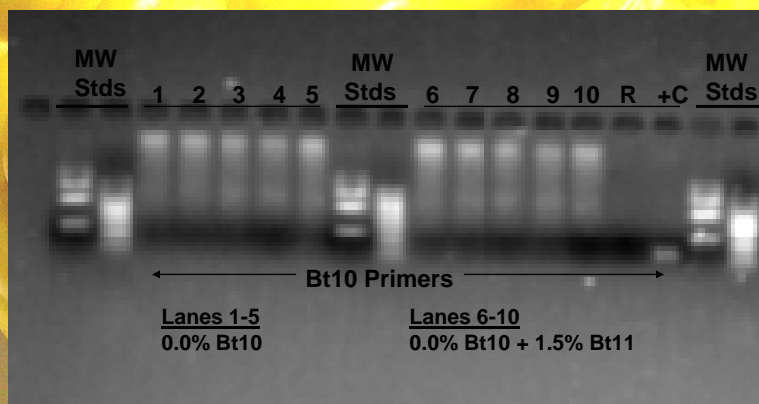
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Evaluating Sensitivity of Bt-10 Method



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Evaluating Specificity of Bt-10 Method



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Summary

- **GIPSA has verified the GeneScan Method to detect the presence of Bt-10**
- **AmpliTaq Gold seems to be more Sensitive compared with Qiagen to Magnesium concentrations**
- **The Qiagen method has more non-specific Amplified products compared with AmpliTaq Gold**

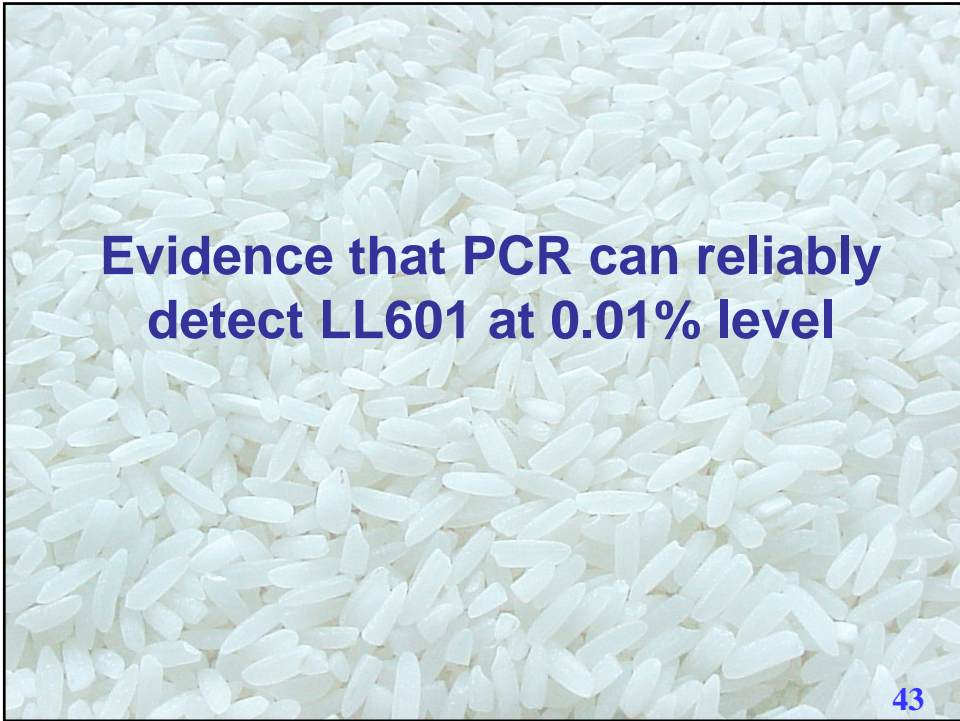
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Inadvertent Release of LLRICE 601

- **Bayer Crop Science Notified USDA of a low level presence of LL601 into US grain exports**
- **GIPSA Biotech Workgroup Performed Sensitivity and Specificity tests of LL601 using both construct and event specific testing methods**
- **GIPSA in cooperation with Bayer Crop Science implemented a proficiency testing program to assure that rice markets have confidence in test results for samples containing LLRICE 601**
- **LL601 contains a 35S promoter and expresses the PAT protein to tolerate the herbicide glufosinate**
- **LL601 construct is similar to other traits that have been government approved for commercialization and pose no human health risks**

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Sensitivity Test LL601 (35S/BAR Method)

Sample (Triplicates)	Results			
	Endogene		Transgene	
	+	-	+	-
1% LL601	3	0	3	0
0.1% LL601	3	0	3	0
0.01% LL601	3	0	3	0
0.005% LL601	3	0	3	0
0.00% LL601	3	0	0	3

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Sensitivity Test LL601 (LL601 Method)

Sample (Triplicates)	Results			
	Endogene		Transgene	
	+	-	+	-
1% LL601	3	0	3	0
0.1% LL601	3	0	3	0
0.01% LL601	3	0	3	0
0.005% LL601	3	0	1	2
0.00% LL601	3	0	0	3

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Sensitivity Test Parboiled LLRICE601 (LL601 Method)

Sample (Triplicates)	Results			
	Endogene		Transgene	
	+	-	+	-
0.1% LL601	3	0	3	0
0.04% LL601	3	0	3	0
0.01% LL601	3	0	3	0
0.00% LL601	3	0	0	3

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Repeatability Test LLRice601 (35S/BAR Method)

Sample	Results			
	Endogene		Transgene	
	+	-	+	-
0.01% LL601 (Twenty Replicates)	20	0	20	0
0.00% LL601 (Two Replicates)	2	0	0	2

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Repeatability Test LLRICE601 (601 Method)

Sample	Results			
	Endogene		Transgene	
	+	-	+	-
0.01% LL601 (Twenty Replicates)	20	0	20	0
0.00% LL601 (Two Replicates)	2	0	0	2

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**USDA/GIPSA
Rice Proficiency Results 35S/BAR Method
(September 2006-April 2008)**

Number of Labs	6
Number of Tests	870
% Correct	100.0%
Provided (+)*	592
False Negatives	0
Provided (-)	278
False Positives	0

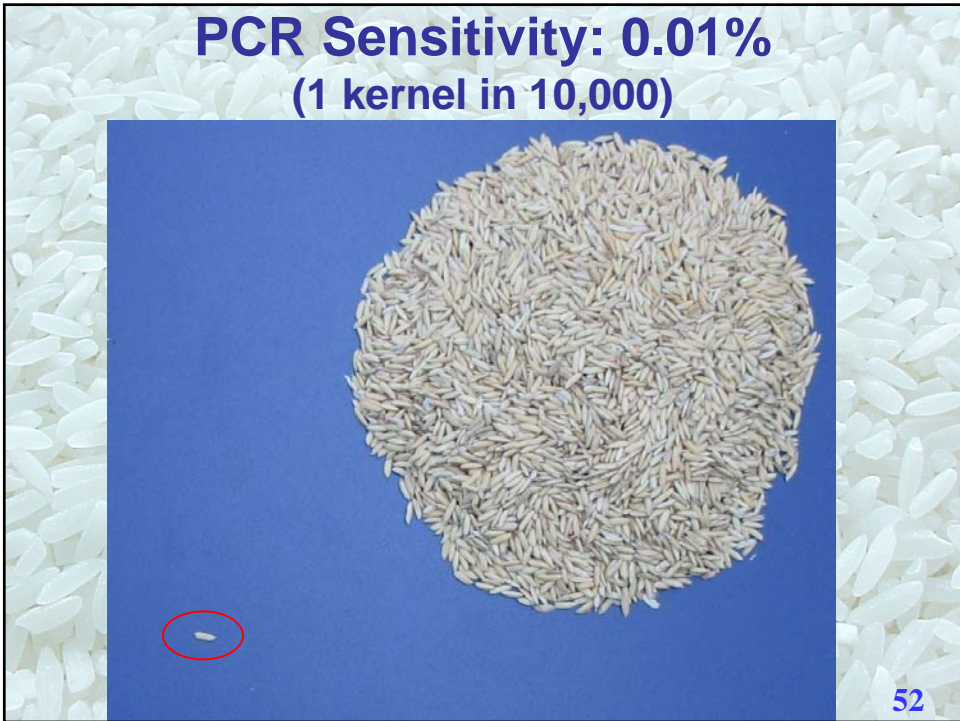
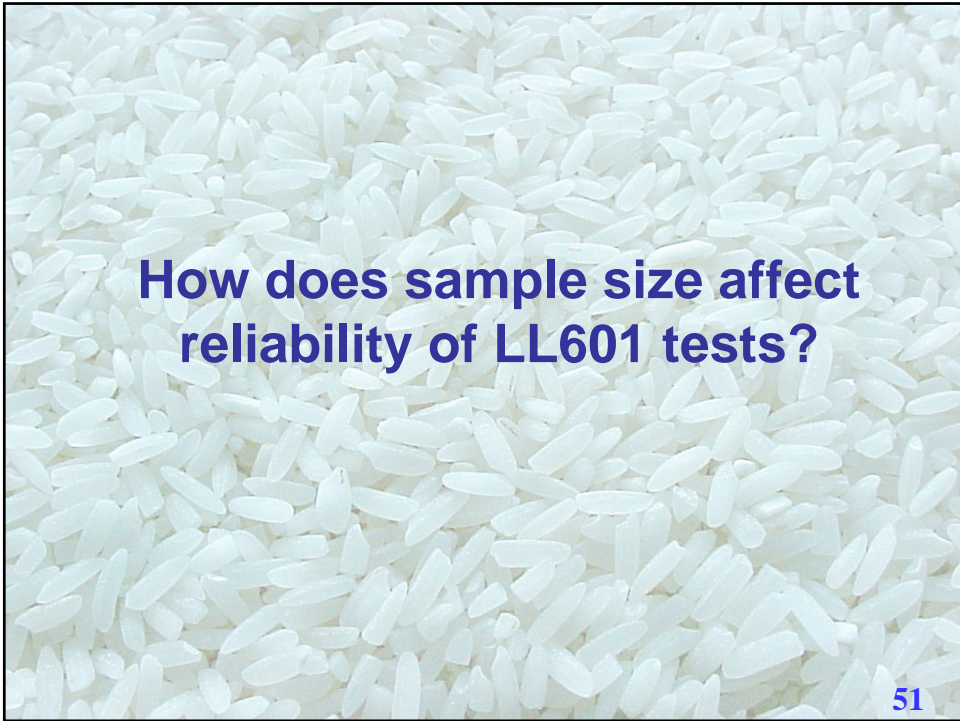
***Positive Samples fortified at 0.03% LL601 or 0.03% LL62**

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**Conclusion regarding PCR
method sensitivity for LL601**

- **Both the 35SBAR method and the LL601-specific method detect (with high confidence) LL601 levels of 0.01% or higher.**

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Increase confidence by analyzing multiple 10,000 kernel (250 gm) portions



95% Probability of Detection

Sample Size (kernels)	Lot Concentration
1,000	0.3
2,500	0.12
3,000	0.1
10,000	0.03
30,000	0.01
50,000	0.006

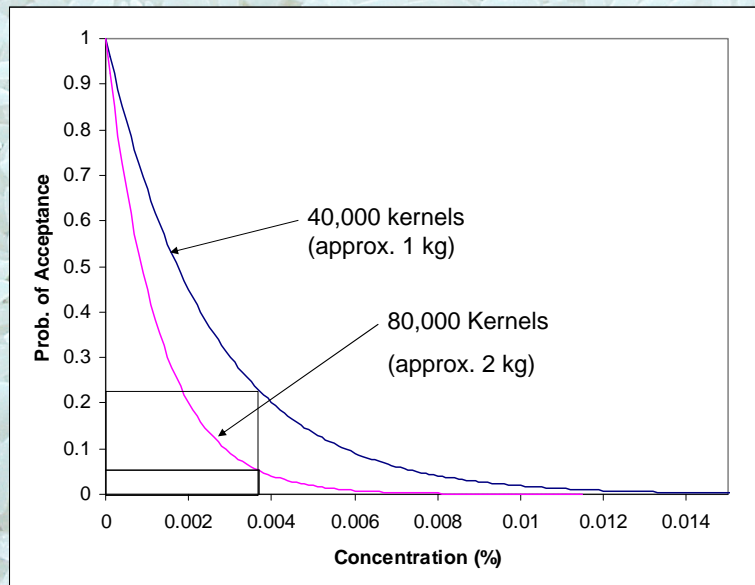
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99% Probability of Detection

Sample Size (kernels)	Lot Concentration
1,000	0.46
2,500	0.19
3,000	0.15
10,000	0.05
30,000	0.015
50,000	0.009

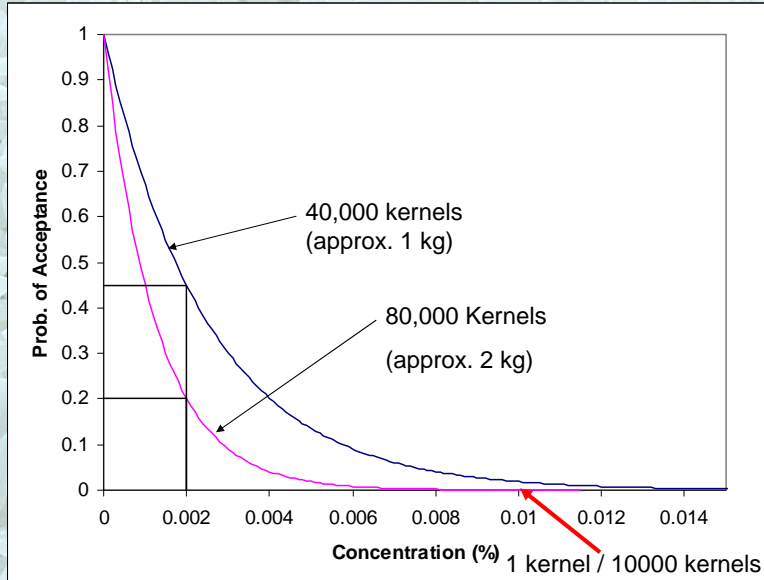
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Probability of Acceptance 40,000 and 80,000 Kernel Samples



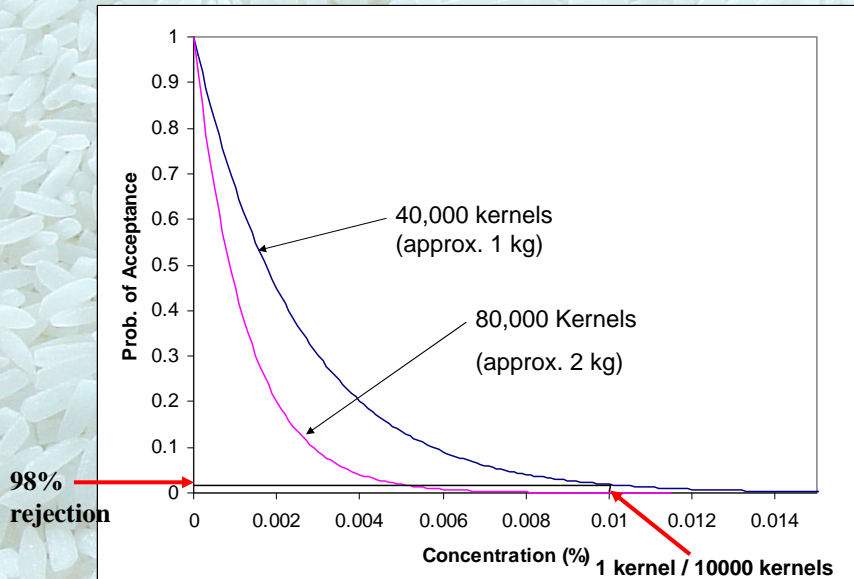
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Probability of Acceptance 40,000 and 80,000 Kernel Samples



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Probability of Acceptance 40,000 and 80,000 Kernel Samples



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Conclusions

- **The 35SBAR and LL601-specific methods can reliably detect one or more LL601 kernels in 10,000.**
- **Sample size and number of samples determine level of risk of lot rejection subsequent to prior acceptance.**
- **Seed test protocols should specify sample size and number of portions to be tested.**

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Thank You



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