

Adventitious Presence defined....

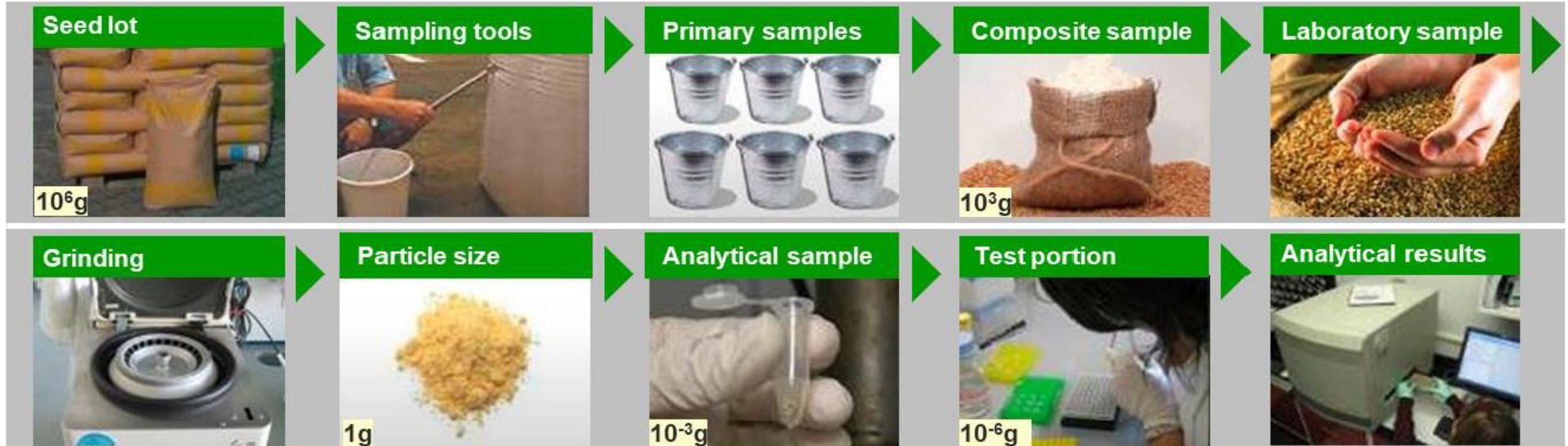
- unintended presence of unwanted biotech traits in seed or grain lots or field populations.
- Other industries – GMO percent



Seed

- Focus on Seed and material coming out of the field to understand Adventitious Presence
- Milled material and processed food has multiple areas for contamination and complicates analysis

Representative Sampling: From the Field to the Lab



Important questions to develop testing strategy...

- What is the product going to be used for?
 - Food
 - Organic
 - Non-GMO
 - Foreign market
- Is there a required level for the product to be for sale?
 - 1%, 0.9%, 0.01%
- Has it been produced under controlled conditions?
 - Beginning seed tested
 - Isolation distances
 - Clean equipment

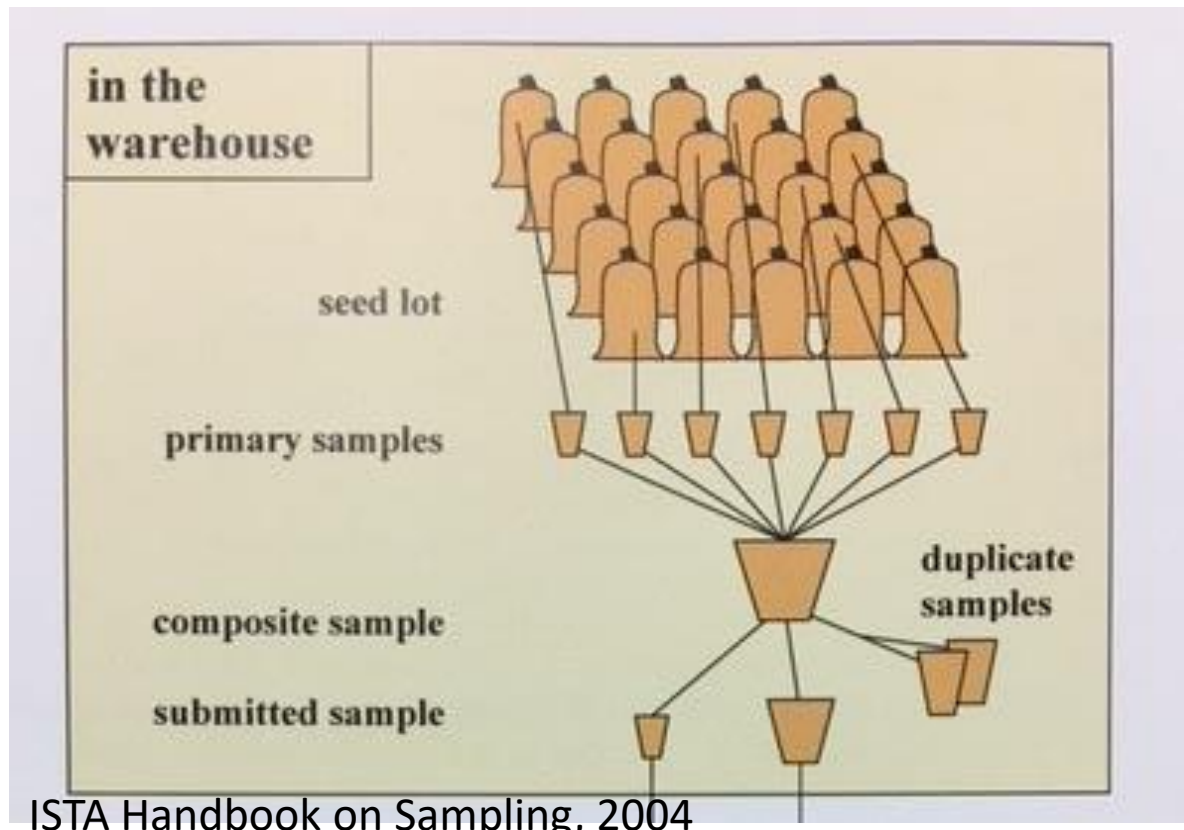
Definitions to keep in mind

- Limit of detection – lowest level detectable but not quantifiable
 - 3x std dev of the blank
- Limit of quantification – lowest amount of detectable analyte that you can detect and quantify
 - 10 x std dev of the blank
 - Or use std curve

SAMPLING IS KEY

(largest source of variation)

No matter how accurately an analysis is made, it can show only the quality of the sample submitted; therefore, it is the responsibility of the seed sampler to assure that the sample is representative of the seed lot.



ISTA Handbook on Sampling, 2004

Capability of the Test

QuickComb Kit for corn bulk grain

LOD (Limit of Detection): Varies by analyte, from 0.25% (1 kernel in a pool of 400) to **1% (1 kernel in a pool of 100).**

If NEED to detect **0.5% LEVEL, THIS DOES NOT WORK** or adjust test to 50 Seeds/ Pool...

Recommend to grind in pools of 800 seed

1 semi load of corn = 54,000,000 kernals

1 test represents 0.0015% of the load

1 sample for every 66,666 seeds in the trailer....



Influence of factors outside the seed

- Sampling – Largest variation
 - Sample submitted
 - Sample ground
 - Sample pulled for testing
 - DNA or Protein Extract pulled for testing
- Extraction of protein
 - Extraction buffer to lateral flow strip
- Pollen dust, particles of contaminating seeds

Testing Methods

- Adventitious Presence Herbicide Bioassay
- Lateral Flow Strips with Ground Material
- DNA methods

AP -1200 or 2400 seed
Most accurate number of
non-trait %



Adventitious Presence (AP) of GMO events.

Substrate Bioassays of 400 to 2400 seeds

Goal is to determine if seed lot (organic or conventional hybrid/cultivar has contamination of transgenic herbicide traits.

Positive and negative check seeds on each

Tray to ensure the presences of herbicide.

% GMO contamination is calculated as the live germinating seeds with the trait. i.e.

3/2400 seeds had glyphosate trait or .125%

Select Tests... ✕

Test Groups:

Bin-GT-WFS (SG,TC-Glyp(RR2),AA)

	<u>Number Of Seeds</u>	<u>Test Instructions</u>
50F Cold <input type="checkbox"/>	-- Select -- ▾	
AA <input type="checkbox"/>	-- Select -- ▾	
AP-2,4-D (ENLIST) <input type="checkbox"/>	-- Select -- ▾	
AP-Dicam <input type="checkbox"/>	-- Select -- ▾	
AP-Gluf (LL) <input type="checkbox"/>	-- Select -- ▾	
AP-Glyp (RR2) <input type="checkbox"/>	-- Select -- ▾	
AP-Isox (BB) <input type="checkbox"/>	-- Select -- ▾	
AP-Isox LFS (BB) <input type="checkbox"/>	-- Select -- ▾	
File <input type="checkbox"/>	-- Select -- ▾	
Germ <input type="checkbox"/>	-- Select -- ▾	
GTSTK <input type="checkbox"/>	-- Select -- ▾	

AP Seed for Insect Traits

294643
CORN
COMPANY CONTACT NOT SET
605-692-2758
(AP-Glut-1200,AP-Glyp-1200,A
V: AP Seed
L: 1

In-House Sample Remarks:

Report Remarks

CORN

Common Scientific

KB Kalyn Brix
Evaluator 2
Logout

Previous Next
Find Recall
Num Seeds Missed Eval

AP-Glut (1200)

TRAIT	2	2	4
N-T	598	598	1196

Trait Percent: 0.33%

+/- Check Seed Options
 Valid Invalid

Test Options EDIT

AP-Glyp (1200)

TRAIT	4	4	8
N-T	596	596	1192

Trait Percent: 0.67%

+/- Check Seed Options
 Valid Invalid

Test Options EDIT

AP Seed (1200)

	1	2	3
Cry1A	NEG	NEG	NEG
CP4 EPSPS	NEG	NEG	NEG
Cry3Bb	POS	NEG	NEG
Cry1F	NEG	NEG	NEG
PAT	NEG	NEG	NEG
Cry34Ab	NEG	NEG	NEG
mBtCry3A	NEG	NEG	NEG
Cry2A	POS	NEG	NEG
Vip3A	NEG	NEG	NEG
ecry3.1	NEG	NEG	NEG

Number Of Seeds 1200

Number Of Reps
 1 2 3

Confirm All Negative 0.12 %

Seed Calc Estimate of

Trait Percent: ----%

+/- Check Seed Options
 Valid Invalid

Test Options EDIT



2 pools of 600 seed
3 pools of 800 seed

AP Seed Report

TRAIT: 0.33%

AP-Gluf

TRAIT: 0.67%

AP-Glyp

AP Seed

From the submitted sample, herbicide bioassays were performed for Glyphosate and Glufosinate tolerance. Result: 8 / 1200 seedlings or 0.67% were positive for Glyphosate (RUR NK603, GA21) tolerance. 4 / 1200 seedlings or 0.33% were positive for Glufosinate (Liberty Link T25) tolerance. For other transgenic traits, protein was extracted from 1200 seeds (2 subsamples of 600 seeds each) and tested using the TraitChek kit AgraStrip Corn Comb w / VIP3A kit by Romer Labs. Result: 1 / 2 subsamples were positive for Cry3Bb (YieldGard RW), 0 / 2 subsamples were positive for Cry1A (YieldGard), 0 / 2 subsamples were positive for Cry1F (Herculex I), 0 / 2 subsamples were positive for Vip3A (Viptera), 0 / 2 subsamples were positive for Cry34Ab (Herculex RW), 1 / 2 subsamples were positive for Cry2A (YieldGard VT), 0 / 2 subsamples were positive for mBtCry3A (Agrisure RW), 0 / 2 subsamples were positive for ecry3.1 (DURA). Using this test result, the test kit limits of detection and the Seed Calc 8 statistical tool estimates the level of AP is 0.12% or less for non-herbicide traits. For all traits tested, the test kit limits of detection and the Seed Calc 8 statistical tool estimates the level of AP to be 0.67% or less.

Grind, Grind, Grind

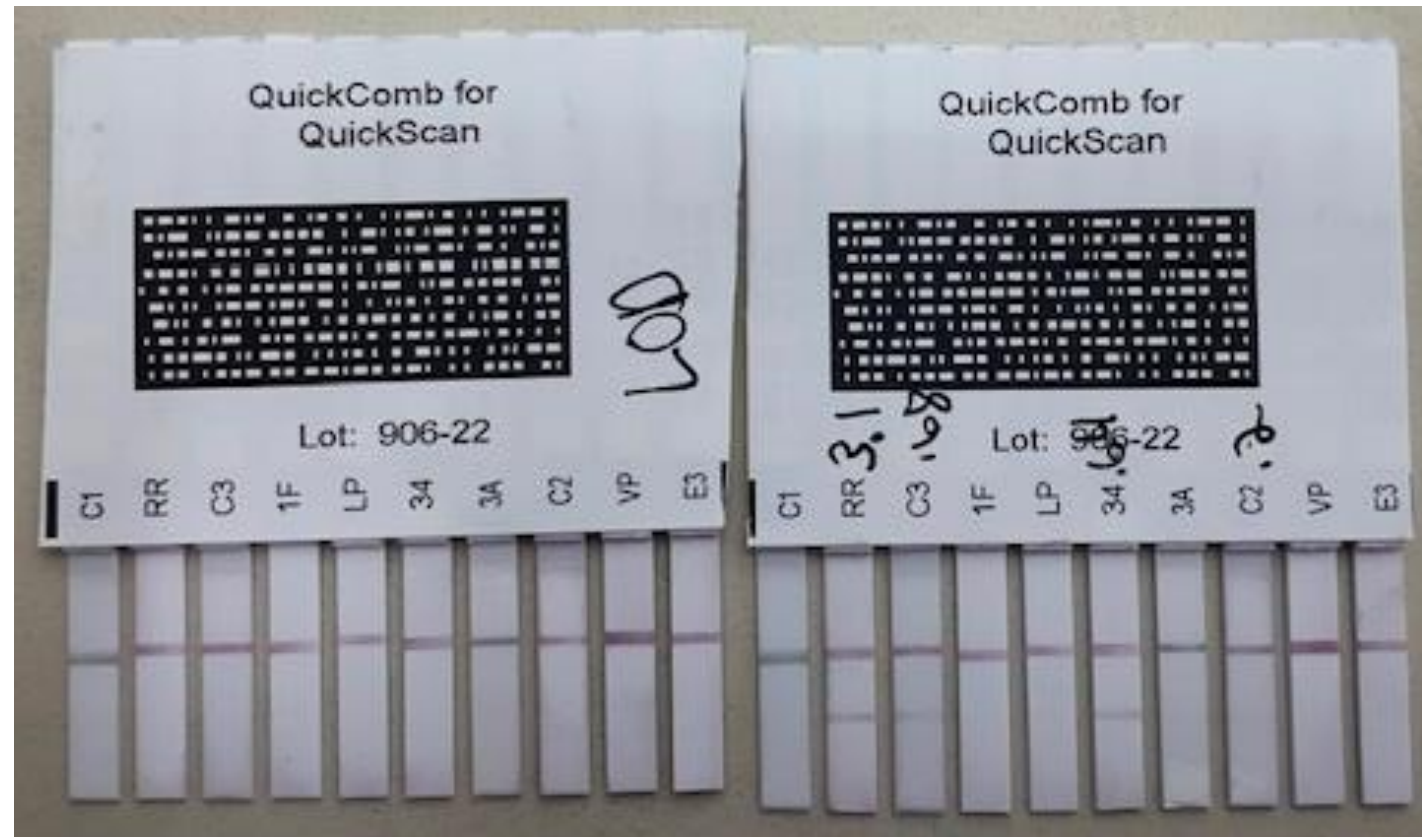
- Protein and DNA testing methodologies
- Grind size can impact results
- Number of particles in 240 grams (represents 800 corn seeds)
 - Have the ability when subsampling to attain a representative sample from each seed
- STRF study on Particle Size influence in results across labs



AP by Lateral Flow Strip Tests

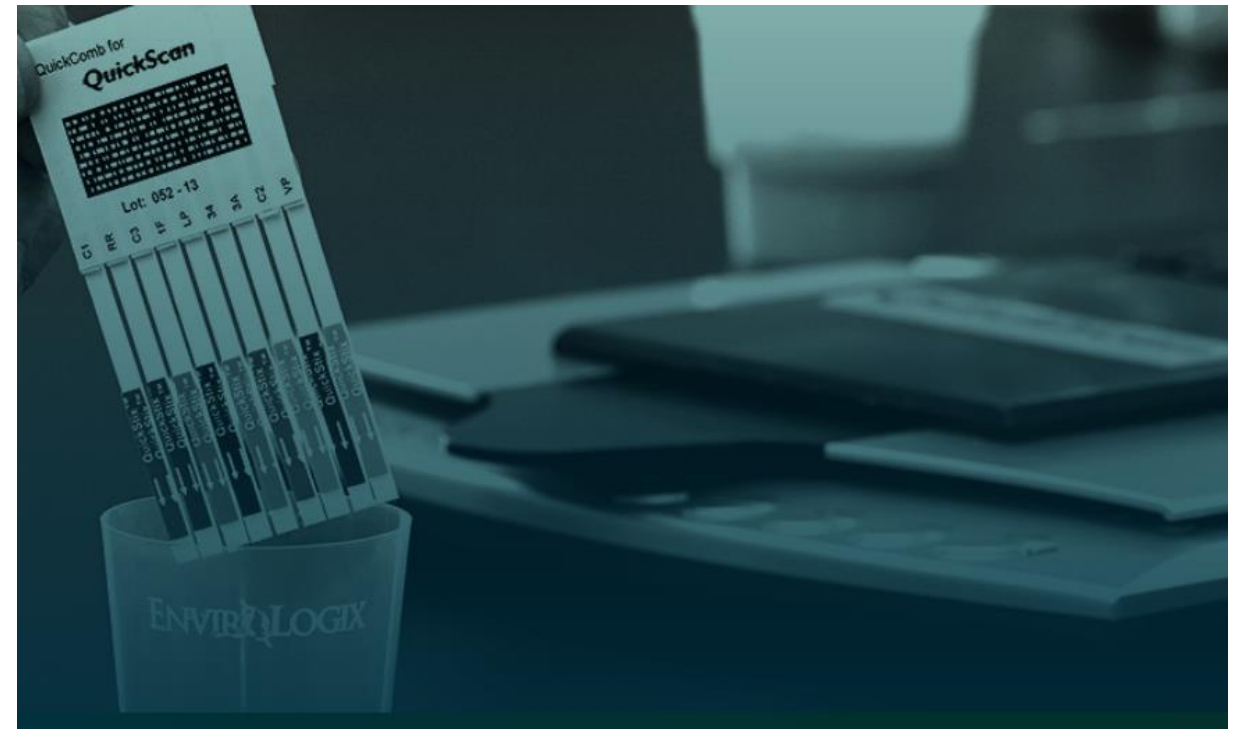
- Cotton and Corn
- Insect Traits and Herbicide traits
- Expression of protein

- Validation



Quantitative ELISA with Lateral Flow Strip tests

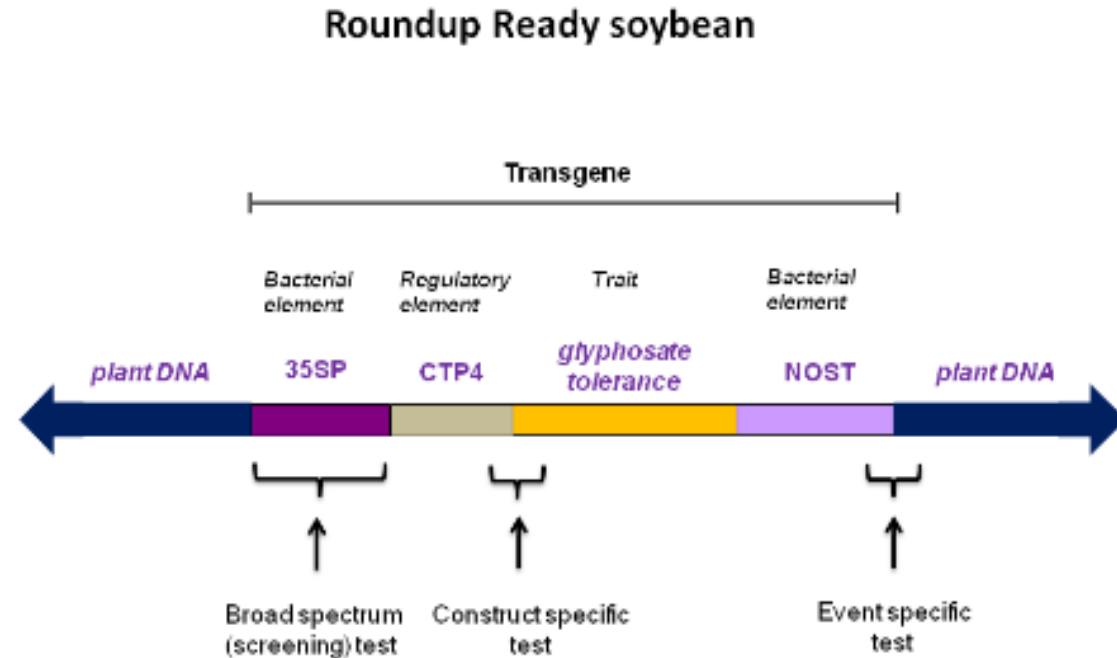
- Envirologix Quik Scan
- 10 traits
- Trait line read by
- LFS challenged with stacked traits



Sample ID	Supplier	Comment 1	Comment 2	Action	Test Kit	Analyse	Result(%)	TL	CL	Lot
1				Accept	AQ-036 TC 13-A	C1: Cry1Ab	< LOD			297
2						RR: CP4 EPSPS	>5.0			
3						C3: Cry3Bb	< LOD			
4						1F: Cry1F	< LOD			
5						LP: PAT/pat	< LOD			
6						34: Cry34	< LOD			
7						3A: mCry3A	< LOD			
8						C2: Cry2A	3.7			
9						VP:VIP3A	< LOD			
10					GMO Corn Sum =		> 8.70			

DNA Detection

- **Event Specific:** Detection of the sequence containing the plant DNA and the transgene. Most used for GMO identification/internationally agreed method due to high specificity.
- **Element Specific:** Detection of the common inserted bacterial elements used in transgenes, such as 35S and TNOS. Typically used for GMO Screening.
- **Construct Specific:** Detection of the junction between two elements. Can be used for screening.



EURL GMFF at JRC

- Hosted at the Joint Research Center (JRC)
- Validates detection methods for the EU
- Provide legal and document guidance for the EU
- Houses and provides positive and negative controls for GM Events
- Proficiency testing, training and workshops
- Maintains a GM Matrix across species and detection methods.



<https://gmo-crl.jrc.ec.europa.eu/>

Matrix Example

GM Event	PCR Screen Sequence					
	35S	NOS	CP4-ESPS	FMV	Bar	Pat
TC1507 Maize (DAS-01507-1)	2	0	0	0	0	2
MIR604 Maize (SYN-IR604-5)	0	2	0	0	0	0
MON810 Maize (MON-00810-6)	2	0	0	0	0	0
MON88017 Maize (MON-88017-3)	2	2	1	0	0	0
NK603 Maize (MON-00603-6)	2	2	1	0	0	0
T25 Maize (ACS-ZM003-2)	2	0	0	0	0	2
MON89034 Maize (MON-89034-3)	2	2	0	2	0	0
Bt11 Maize (SYN-BT011-1)	2	2	0	0	0	2
GA21 Maize (MON-00021-9)	0	2	0	0	0	0
MIR162 Maize (SYN-IR162-4)	0	2	0	0	0	0
MON87460 Maize (MON-87460-4)	2	2	0	0	0	0
DAS-40278-9 Maize (DAS-40278-9)	0	0	0	0	0	0
5307 Maize (SYN-05307-1)	0	2	0	0	0	0
MON 87411 Maize (MON-87411-9)	2	0	1	0	0	0

AP DNA Detection Qualitative

- Results are either positive or negative for the presence of target

Positives:

- Can use common gene specific assays to detect GMO
- DNA Based test is more sensitive than LFS
- Recognized world wide for standard test method for GMO
- Can be less expensive than rtPCR
- Electronic results can be stored

Negatives:

- Follow up testing needed to know specific contaminate
- More labor intensive than rtPCR
- Requires knowledge of equipment and software
- Requires expensive equipment and reagents
- If using Ethidium Bromide for staining it is a carcinogen and mutagen

AP DNA Detection Semi-Quantitative

- Estimated impurity is calculated based on the number of positive pools, the number of total seeds and the primers tested.

Positives:

- Can use common gene specific assays to detect GMO
- Highly sensitive (LOD 0.01 GMO)
- Recognized world wide for standard test method for GMO
- Electronic results can be stored
- Results calculated by software

Negatives:

- Follow up testing needed to know specific contaminate
- Lack of repeatability of exact percentage at low levels.
- Requires replicates of controls and samples for accuracy.
- Requires knowledge of equipment and software
- Requires expensive equipment and reagents

Test	Test Type	Size of Test	Method	Detection Level	Report	Advantages
Herbicide Bioassay and Lateral Flow Strip (LFS) Screen	Quantitative and Semi-Quantitative	2 pools of 600 or 3 pools of 800 seeds	Herbicide Bioassays and protein for insecticide traits	0.12-0.25% or less	Estimated impurity is calculated based on the percentage of trait tolerant seedlings, number of positive pools, the number of total seeds tested.	Quantitative herbicide tolerant results Screen majority of insect traits.
Quantitative Lateral Flow Strip Screen (LFS)	Quantitative	1 pool of 800 seeds or kernels	ELISA LFS comb for both Herbicide and Insecticide Traits	0.9% or less	QuickScan system estimates contamination based on the development of the test line of the lateral flow strip compared to known standards	Fast screen for majority of herbicide and insecticide traits.
PCR Screen	Semi-Quantitative	Equal pools for a total of 3000 or 10000 seed	Detection of specific DNA sequences utilized in transgenic insertions.	0.03-1.34%	Estimated impurity is calculated based on the number of positive pool, the number of total seeds and the primers tested.	Most sensitive analysis and screens for many events with the 35S promotor and TNOS terminator.



An Introduction to SeedCalc

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SeedCalc8

- ISTA Statistics committee: Free Tool!
 - <https://www.seedtest.org/en/stats-tool-box-content---1--1143.html>
- “Seedcalc8 is a Microsoft Excel® application
- Design seed testing plans for purity/impurity characteristics including testing for adventitious presence levels of biotech traits in conventional seed lots.
- Can also be used to estimate purity/impurity in a lot or sample when results are available



ISTA
Seed Quality Assurance

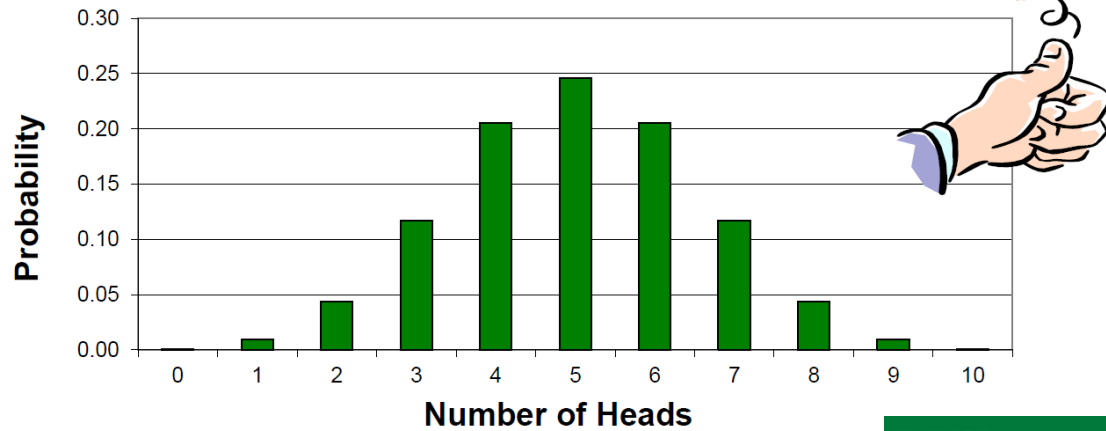
Impurity Estimation & Confidence Intervals (Assay measures impurity characteristic)

(Number of seed sampled should not exceed 10% of total number in population)

# of Seed Pools	15	Computed % in sample	0.03 %
# of Seeds per Pool	200		
Total Seeds Tested	3000		
# Deviants Pools	1	<i>Measured property on seed pools</i>	
		Desired Confidence Level	95 %
Upper Bound of True % Impurity		0.16	
		<i>(95% confident that the lot impurity is below 0.16%.)</i>	
2-sided CI for True % Impurity		0.00	to 0.19
Lower Bound of True % Purity		99.84	
		<i>(95% confident that the lot purity is above 99.84%.)</i>	
2-sided CI for True % Purity		99.81	to 100.00

SeedCalc

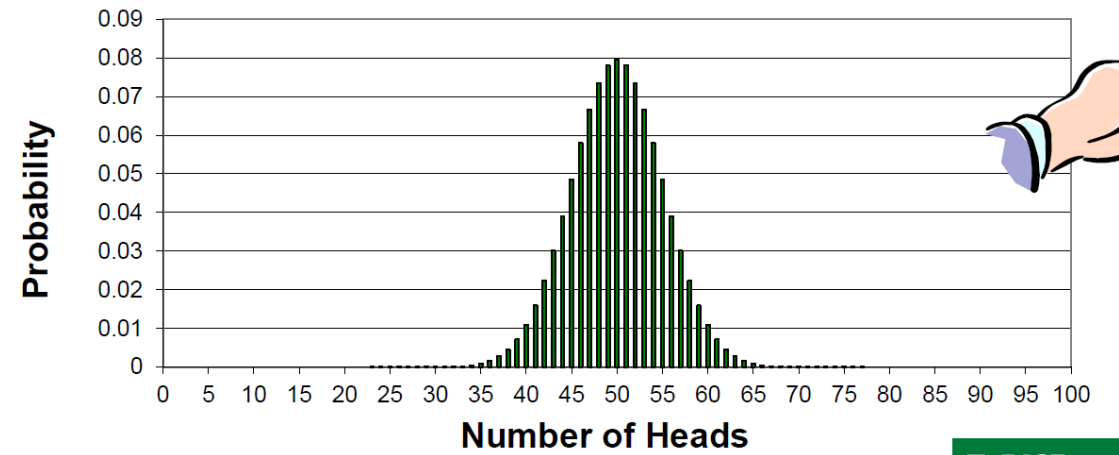
Probability of Tossing X Heads in 10 Tosses



Seedcalc Training - 230323



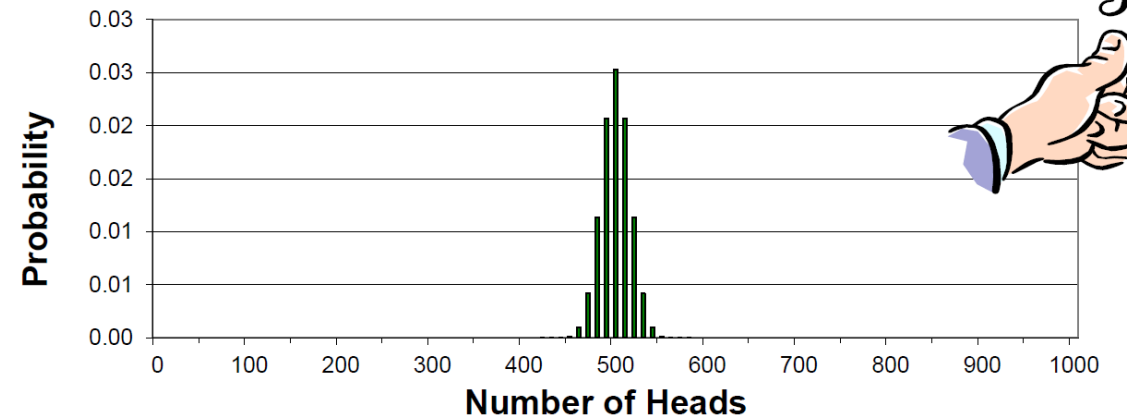
Probability of Tossing X Heads in 100 Tosses



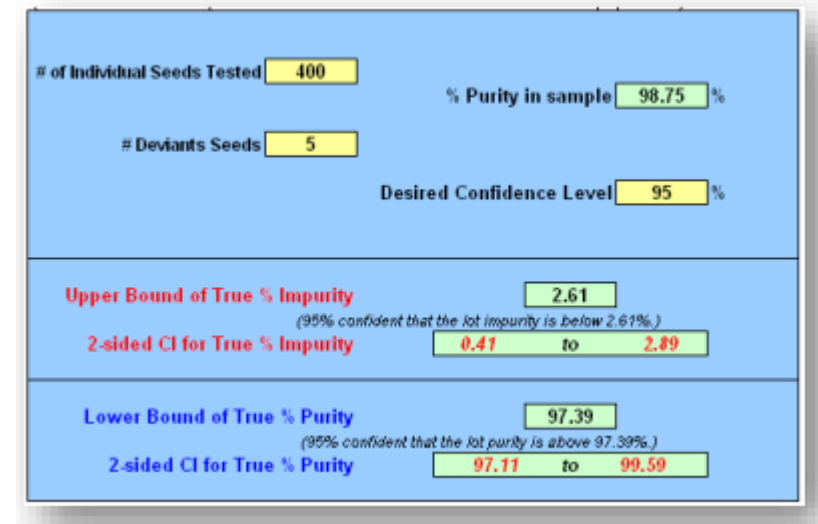
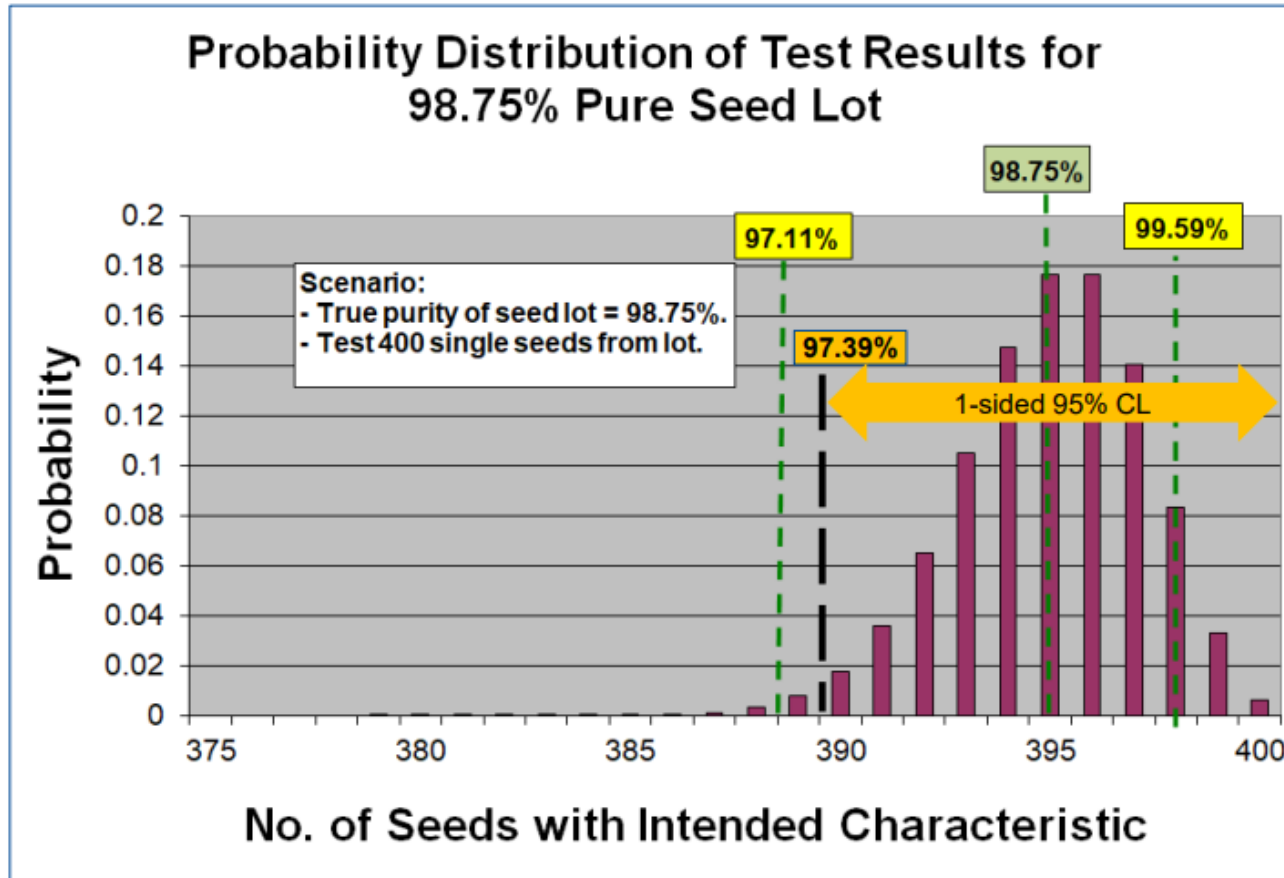
Seedcalc Training - 230323



Probability of Tossing X Heads in 1000 Tosses



Qualitative Purity Estimation: Individual Seeds



Qualitative Impurity Estimation

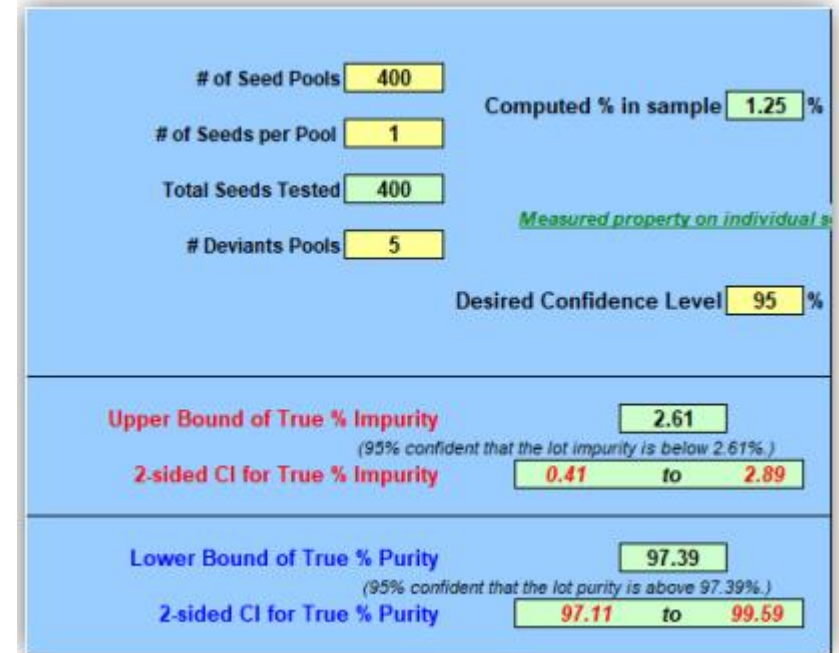
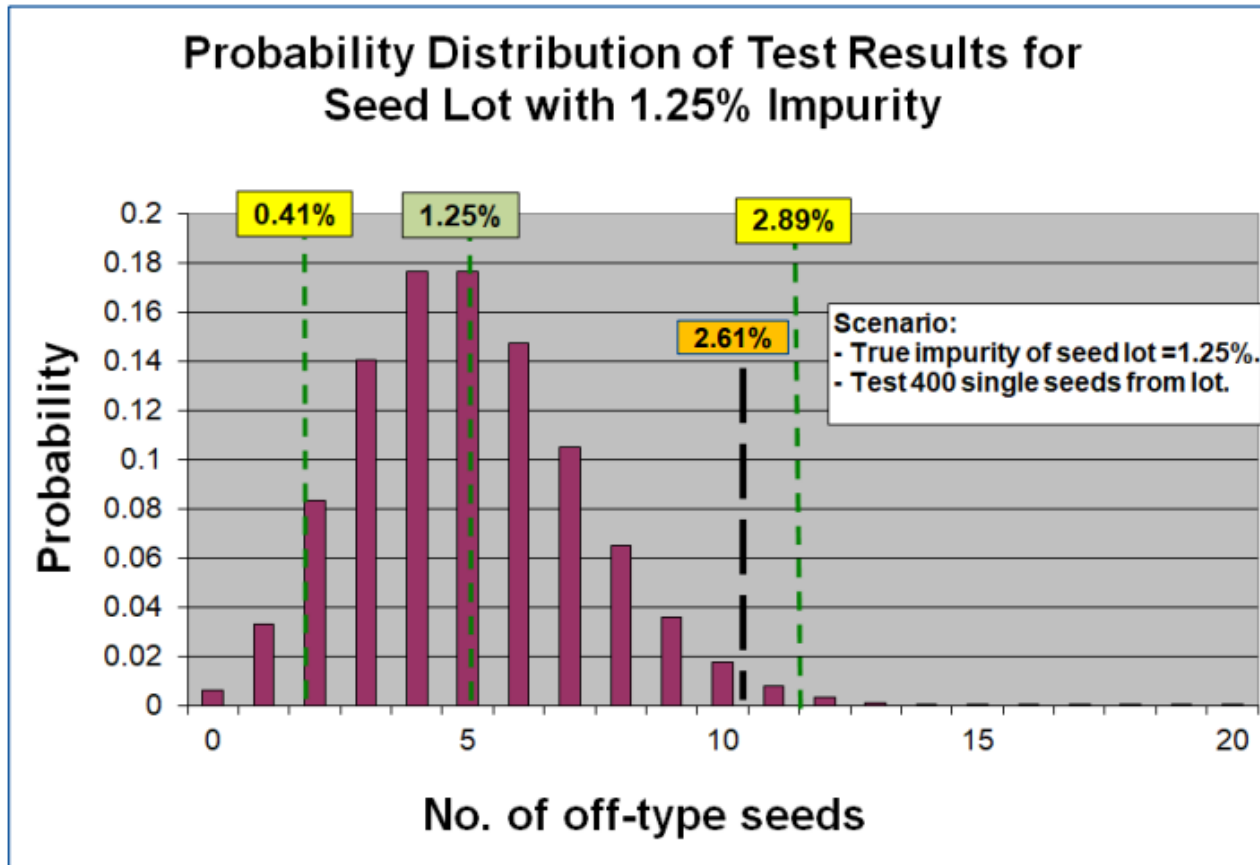
- Designed for handling results from pooled seed, but may also be applied for single seed testing.

# of Seed Pools	400	Computed % in sample	1.25 %
# of Seeds per Pool	1		
Total Seeds Tested	400		
# Deviants Pools	5	<i>Measured property on individual s</i>	
		Desired Confidence Level	95 %
Upper Bound of True % Impurity			2.61
		<i>(95% confident that the lot impurity is below 2.61%.)</i>	
2-sided CI for True % Impurity			0.41 to 2.89
Lower Bound of True % Purity			97.39
		<i>(95% confident that the lot purity is above 97.39%.)</i>	
2-sided CI for True % Purity			97.11 to 99.59

Inputs to SeedCalc

- **Pool** – The tested sample is separated into equal portions and analyzed individually but reported based on the overall sample result.
- **Pool Size** – Number of seeds that are ground at one time
- **Number of Deviants**
- **Confidence Level** – 95% is standard

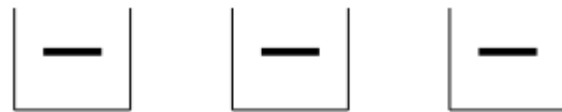
Qualitative **Impurity** Estimation: Individual Seeds



Seed Pooling and Testing Strategies

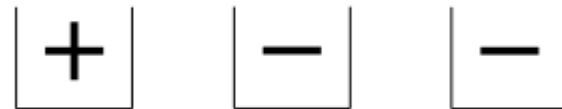
- Save Money and Time compared to single seed testing
- Can use qualitative results on pools to determine quantitative results (Semi-Quantitative analysis)

Lab Sample
3 x 100 seeds



Seed Calc Estimate (%)

Not Detected, Upper Bound <0.99%

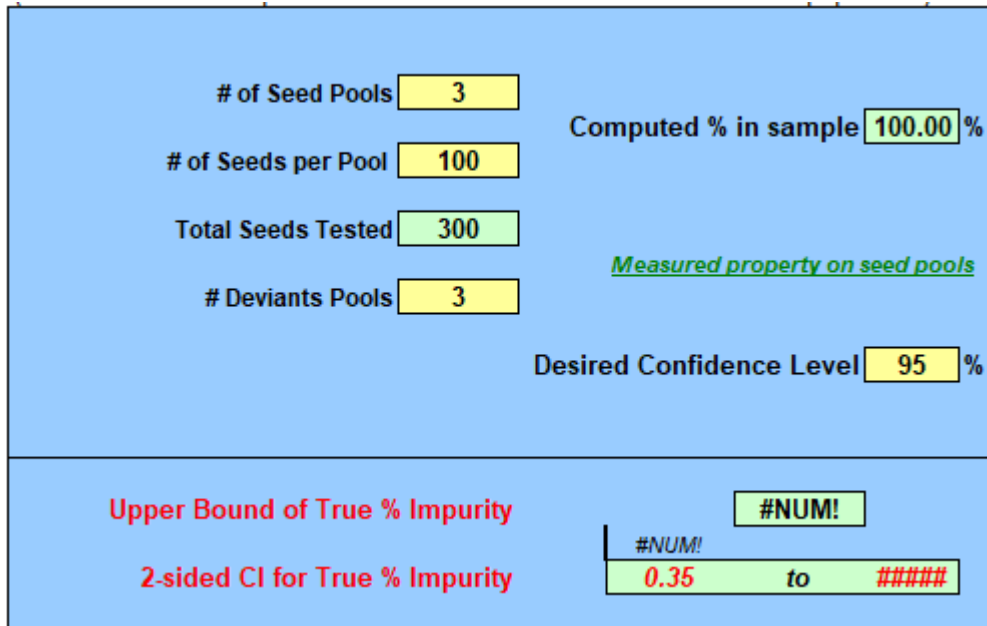


Detected at 0.4%, Upper Bound <1.98%

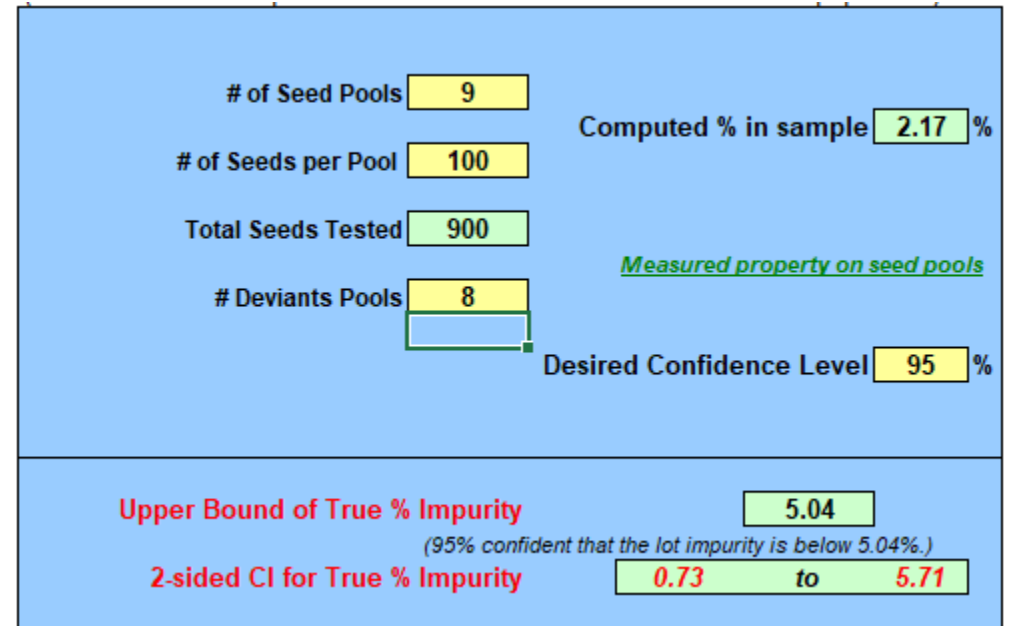


Detected at 1.09%, Upper Bound <4.00%

What if all are pools are positive?



- No statistics can be completed to estimate the % impurity in the sample nor lot.



- Increase number of pools and/or decrease pool size to aim for at least 1 pool with no detection.

Hands On Activity

Number of Pools	# seeds/ pool	# deviants	Computed %	Range
3	8	1		
8	3	1		
3	80	1		
8	30	1		

How to get the best plan?

- Estimate of seed lot impurity
- What level of testing required?
- Other ?

How GMO contamination level impacts pool size

Pool Size # seeds	GMO Contamination Level		
	0.1%	0.6%	1.0%
100	<1	<1	1
200	<1	1	2
300	<1	2	3
500	<1	3	5
1000	1	6	10

The estimated number of transgenic seeds for a given pool size based on theoretical GMO contamination level.

Seed Calc Semi Quantitative Scenarios

Number of Seeds per Pool	Scenario 1		Scenario 2	
	Number of pools	Max Seed Calc Est GMO% with 1 neg pool	Number of pools	Max Seed Calc Est GMO% with 1 neg pool
200	15		6	
300	10		6	
500	6		--	--
1000	3		--	--

Estimated SeedCalc GMO% of different seeds/pool and number of pools with at least 1 negative pool detected.

SeedCalc Exercise 1

- A herbicide tolerant soybean seed lot needs to be certified for purity
 - The lab tested 400 individual seeds using a lateral flow strip
 - The results showed 8 seeds tested negative, 3 seeds were inconclusive, and the remaining seeds tested positive.
 - With a 95% Confidence Interval answer the following:

The purity of the test sample is:

- a) 98.00%
- b) 96.39%
- c) 97.25%
- d) 97.98%

The purity of the seed lot is:

- a) 97.98%
- b) >96.39%
- c) 95.49%
- d) >96.07% and <99.13%

SeedCalc Exercise 2

- A cotton triple event stack needs a seed purity
 - The lab tested 352 individual seeds with event specific TaqMan PCR methods
 - The results showed 10 failed, and the remaining 342 seeds tested positive.
 - With a 95% Confidence Interval answer the following:

The purity of the test sample is:

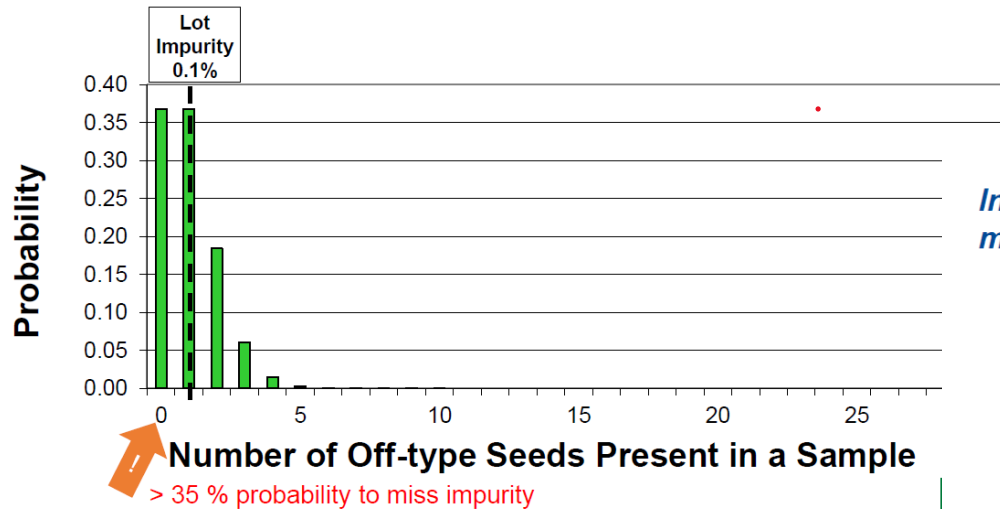
- a) 99.13%
- b) 100.00%
- c) 98.93%
- d) 97.16%

The purity of the seed lot is:

- a) 95.23
- b) 97.16
- c) 94.84
- d) 99.13

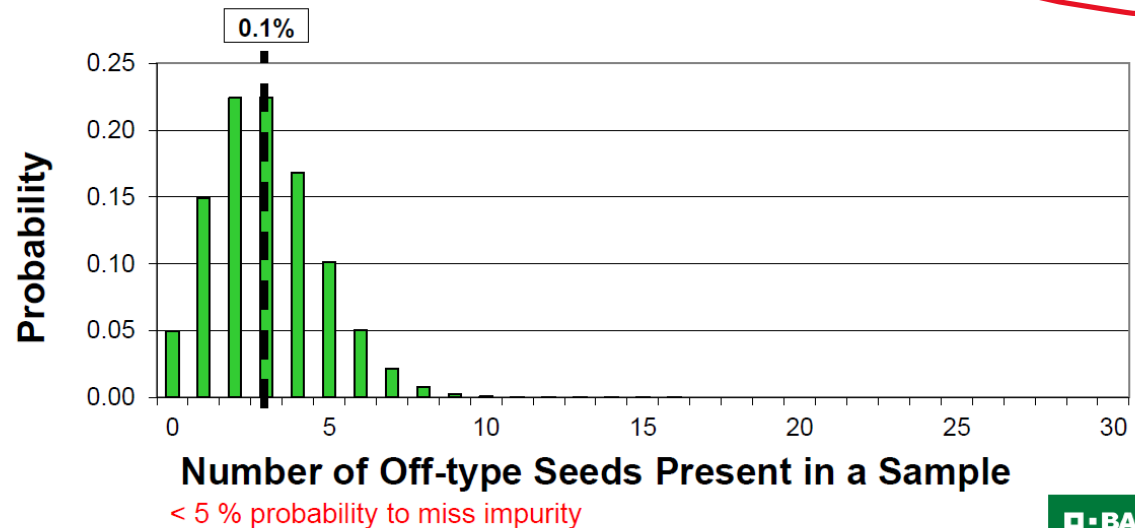
Need to test more than the calculated % due to statistics

What would be the expected result if 1000 seeds are sampled and tested from a seed lot containing 0.1% impurity?



Seedcalc Training - 230323

Increase probability to detect off-types to >95% by testing approximately 3 times more seeds than the set "Zero detect" Threshold. (if 0,1% => test 3000 seeds)



Seedcalc Training - 230323

Seed Lot, Guestimated Contamination level is .5% impurity

- Test at least 3000 seed total
- Capability of test is 1/1000 detection capability
- Testing Strategy?

Pool Size?

Pools

Let's run SeedCalc

Pool Size # seeds	GMO Contamination Level		
	0.1%	0.6%	1.0%
100	<1	<1	1
200	<1	1	2
300	<1	2	3
500	<1	3	5
1000	1	6	10

Upper bound of impurity?

Seed Lot, Guestimated Contamination level is 0.1% impurity

- Test at least 3000 seed total
- Capability of test is 1/1000 detection capability
- Testing Strategy?

Pool Size?

Pools

Let's run SeedCalc

Pool Size # seeds	GMO Contamination Level		
	0.1%	0.6%	1.0%
100	<1	<1	1
200	<1	1	2
300	<1	2	3
500	<1	3	5
1000	1	6	10

Upper bound of impurity?

Seed Lot, Guestimated Contamination level is 0.1% impurity

- Test at least 10,000 seed total
 - Capability of test is 1/1000 detection capability
 - Testing Strategy?
-
- Upper bound of impurity -

Questions?

- Hands on activity for this on Friday this week
- Download SeedCalc 8 from here:

