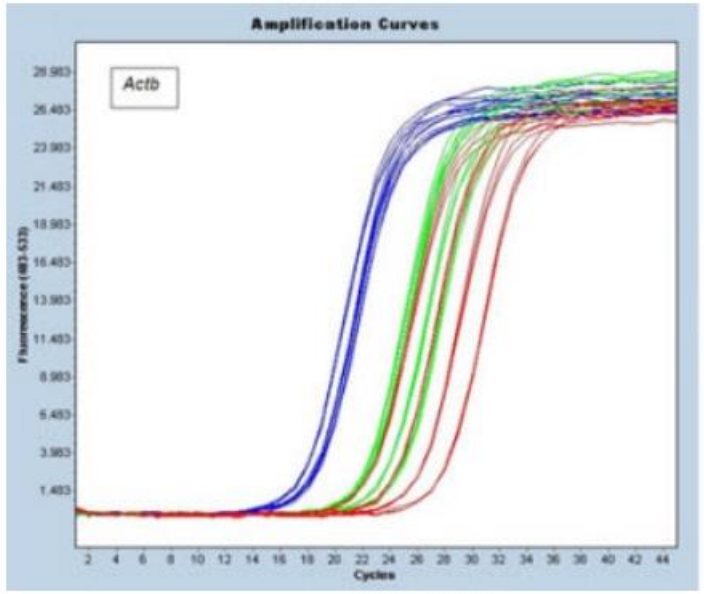
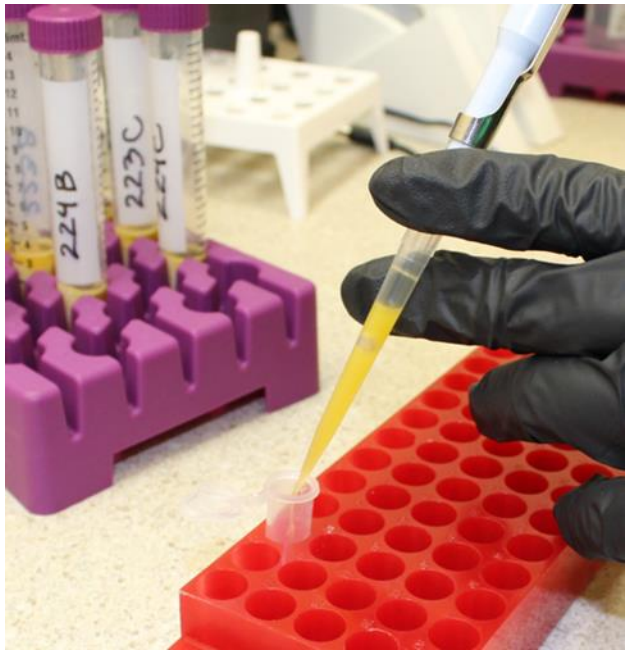
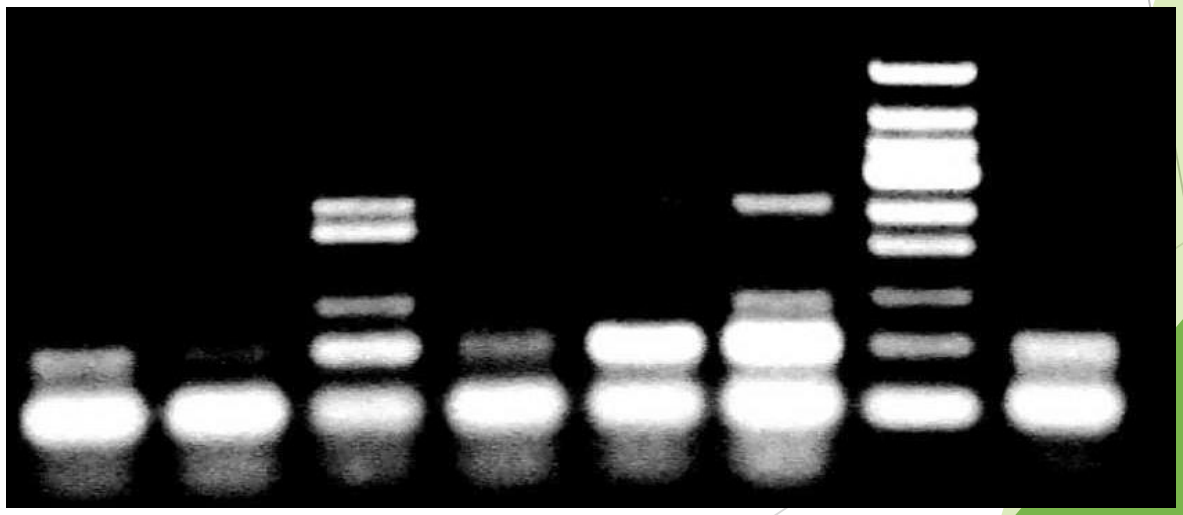


PCR in Seed Testing - Overview and Applications





Overview

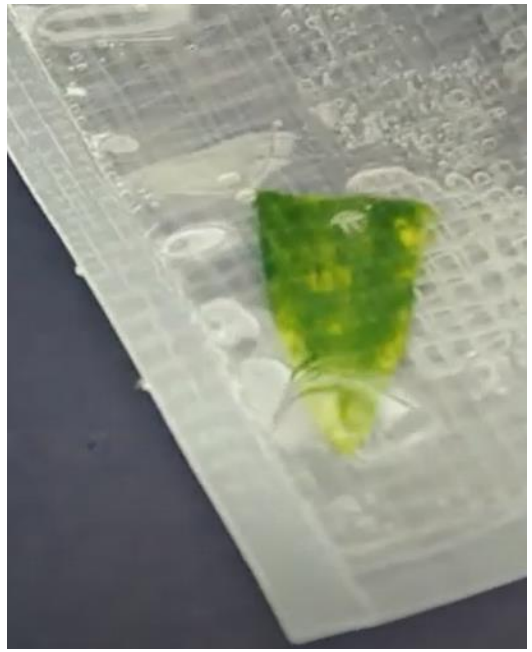
- What is PCR, what types of PCR , how does it work?
- Methodology of various PCR types.
- Applications in seed testing.
- AP Testing in seed protocols
- Discussion of hands-on PCR

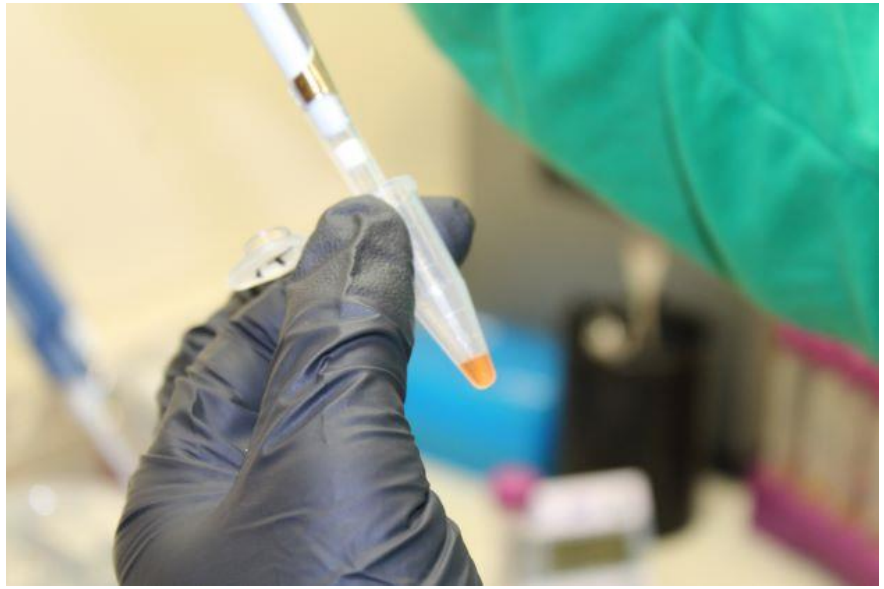
PCR Overview

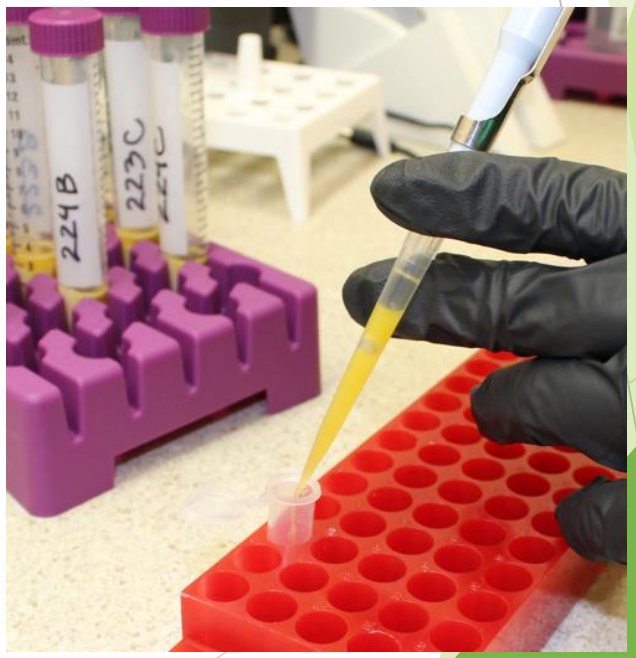
- Polymerase Chain Reaction: invented in 1985 by Karry Mullis
- Involves amplification of template DNA in a reaction to produce millions (or more) copies.
  - Can increase copy number from undetectable to easily detectable levels in a matter of hours
- Used in medical, diagnostic, ag., basic research, etc.
- Reagent Required: template DNA, primers, nucleotides, thermostable polymerase, cofactors
- Equipment Required: Thermal cycler, pipettes, reaction tubes of some sort, equipment to visualize DNA (light box if gel or real time thermal cycler detecting fluorescence)

PCR Components - DNA

- DNA template – trace amount of DNA, could from any source.

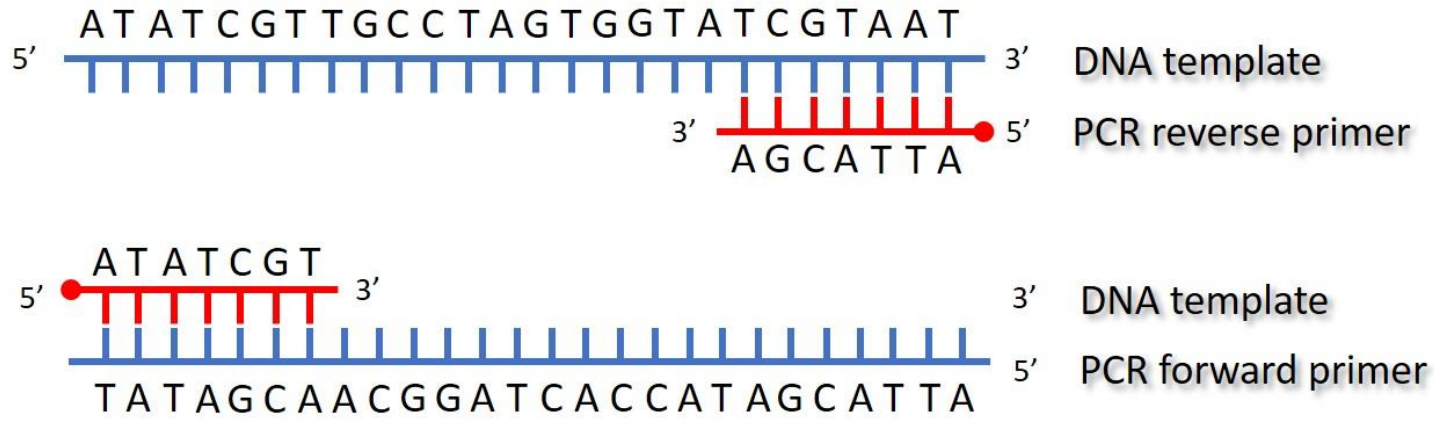






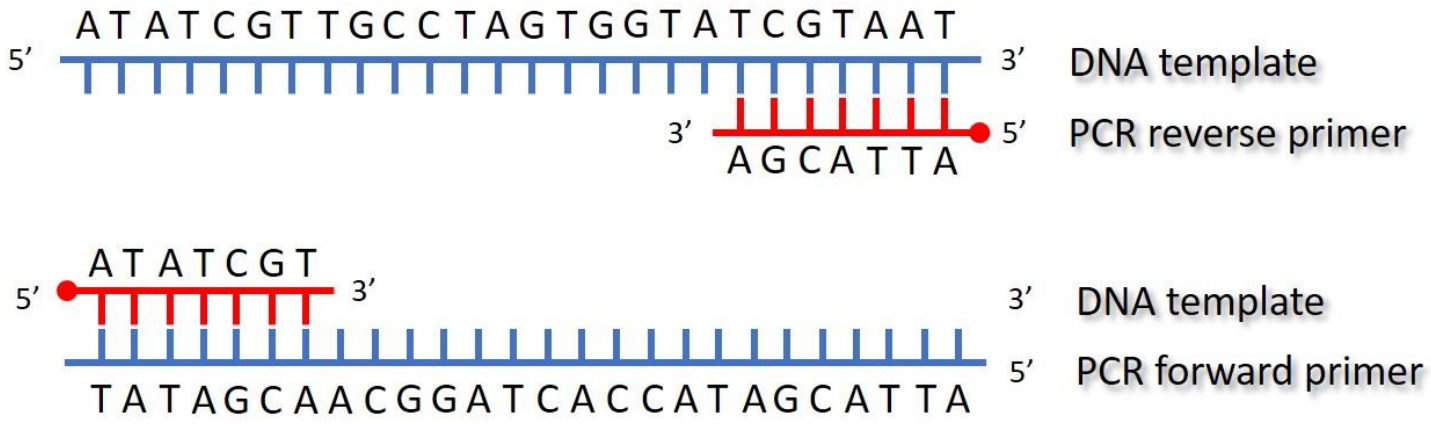
PCR Components - Primers

- Primers – Single strand DNA sequences complementary to template DNA to be amplified. Observe base pair “binding rules”



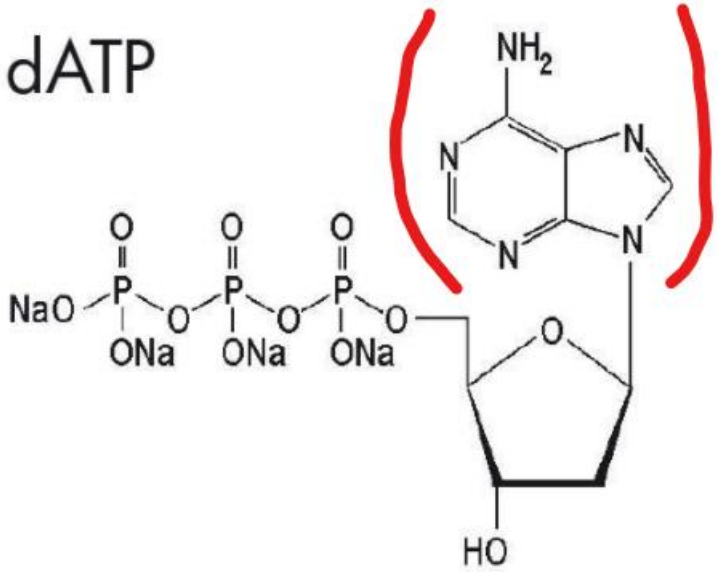
PCR Components - Primers

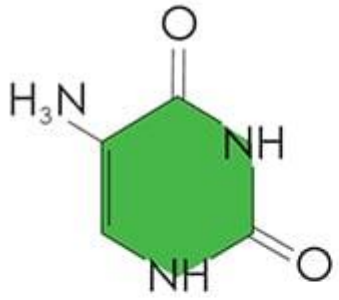
- Many companies produce custom primers, provide design tools to calculate Tm, dimer formation, and other factors that contribute to successful PCR (IDT Oligo Analyzer and PrimerQuest Design Tool)
- Some reactions involve more than two primer; Competitive PCR, KASP, multiplex reactions amplifying one ore more alleles.

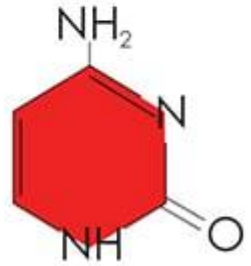


PCR Components - Nucleotides

- Adenine, Guanine, Cystosine, Thymine are the four nitrogenous bases for deoxyribonucleotide triphosphates. These are the building blocks of DNA





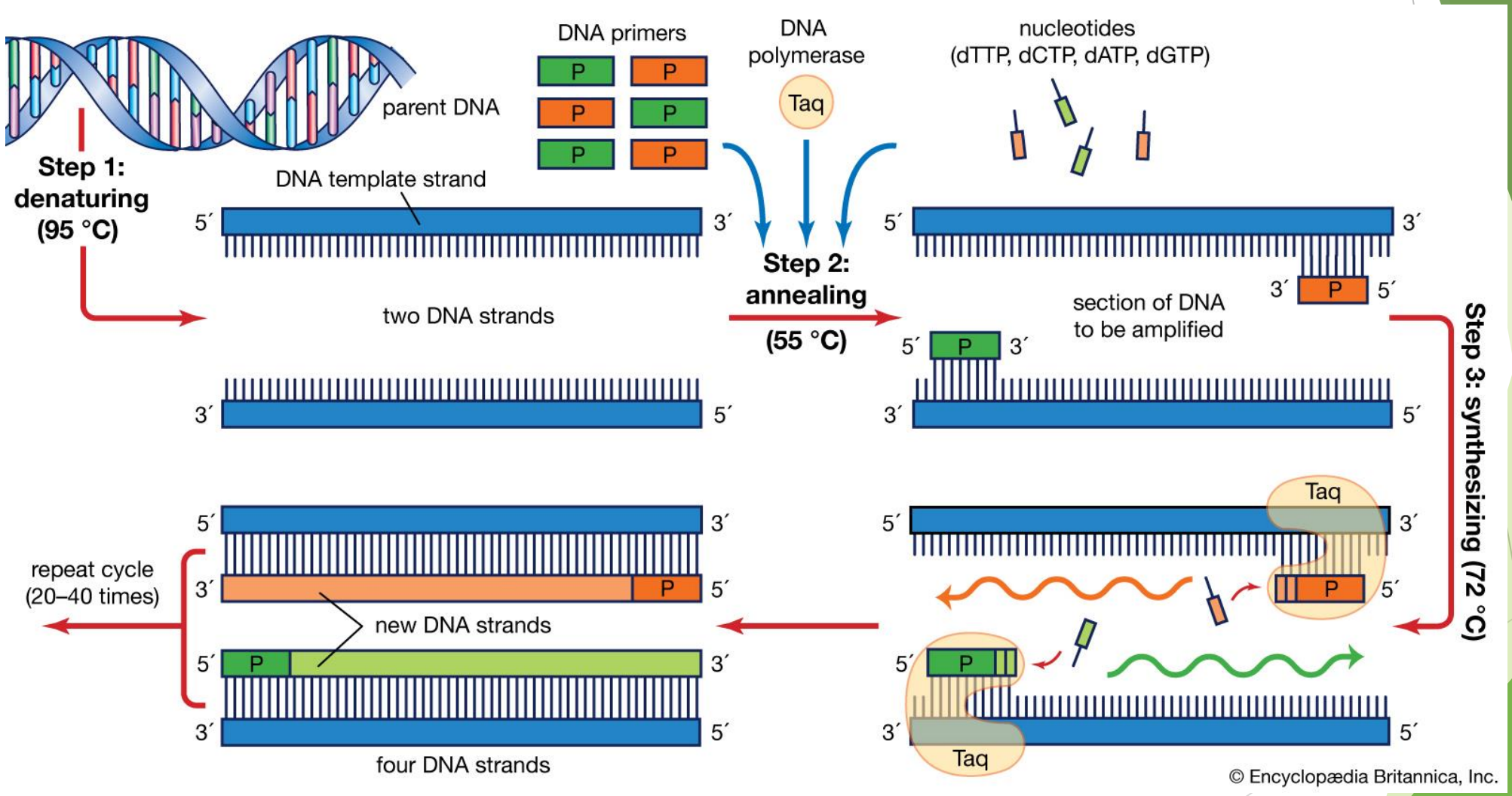


PCR Components - Polymerase

- DNA polymerase – must be thermostable to withstand high temps in PCR; does not denature at 95C (~200F).
- Historically from Thermus aquaticus, Pyrococcus, Thermococcus. Bacteria that live in hot spring environments of ~160F.
- DNA sequence has been cloned to enable mass production of this enzyme in Escherichia coli.
- Cofactors- Mg(2+), Mn(2+)

Taq Polymerase with DNA

Graphical Overview

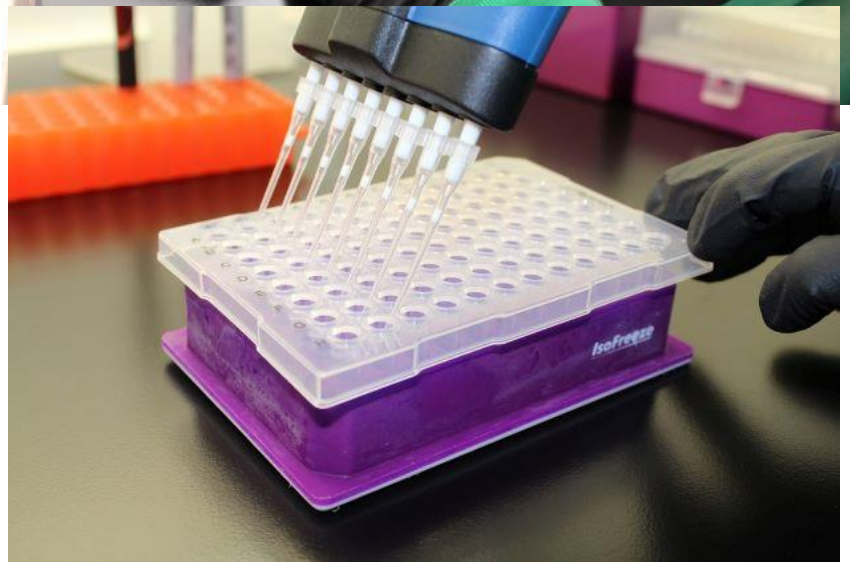


Britannica, The Editors of Encyclopaedia. "polymerase chain reaction". Encyclopedia Britannica, 13 May. 2021, https://www.britannica.com/science/polymerase-chain-reaction. Accessed 22 February 2022.

Video of PCR

- https://dnalc.cshl.edu/resources/3d/19-polymerase-chain-reaction.html

Equipment used for PCR



Uses for PCR in Seed Testing

- Amplification of any DNA of interest to producers, consumers, agronomists, researchers,….anyone.
- GMO events: Can detect any GMO event given you have sequence information
  - May be interested in Adventitious Presence or to confirm event presence (zygosity)
- Example MON88017 (encodes cry3Bb1): could perform Cry3Bb1 ELISA as well, but ELISA does not confirm event, where PCR can
- Example: MON87411 (encodes cry3Bb1, cp4 epsps, dvsnf7). ELISA Cannot detect dvsnf7 since this gene encodes sequence to produce dsRNA only, no protein.
- Agronomic DNA sequences of interest: Certain alleles for disease resistance, yield, maturity, phenotype.
- Pathogen Detection: Fungus, bacteria, virus DNA sequences

End Result of PCR – What's Next?

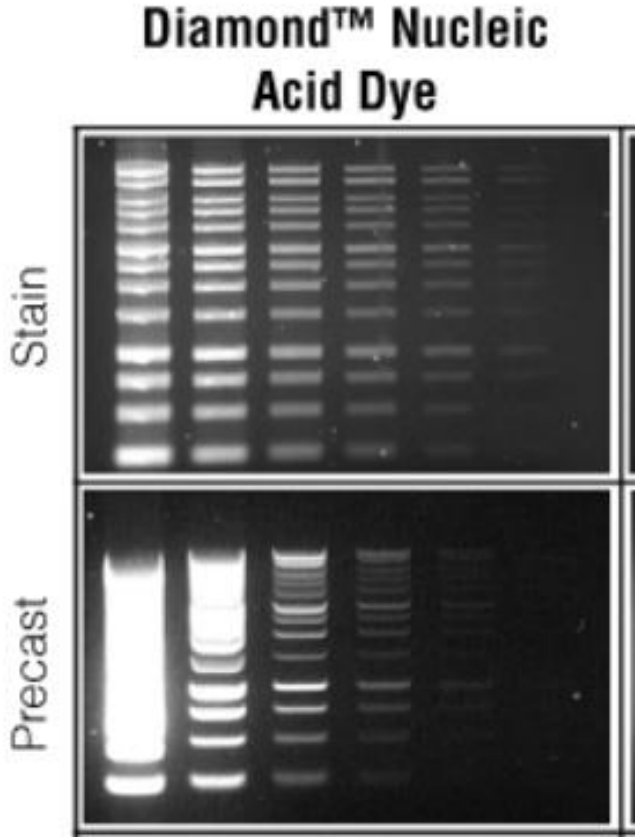
- How do we determine if any DNA was amplified?
- Traditionally end point PCR products are loaded into an agarose gel for electrophoresis and subsequent staining
- For quantitative PCR; fluorescence is measure at each PCR cycle

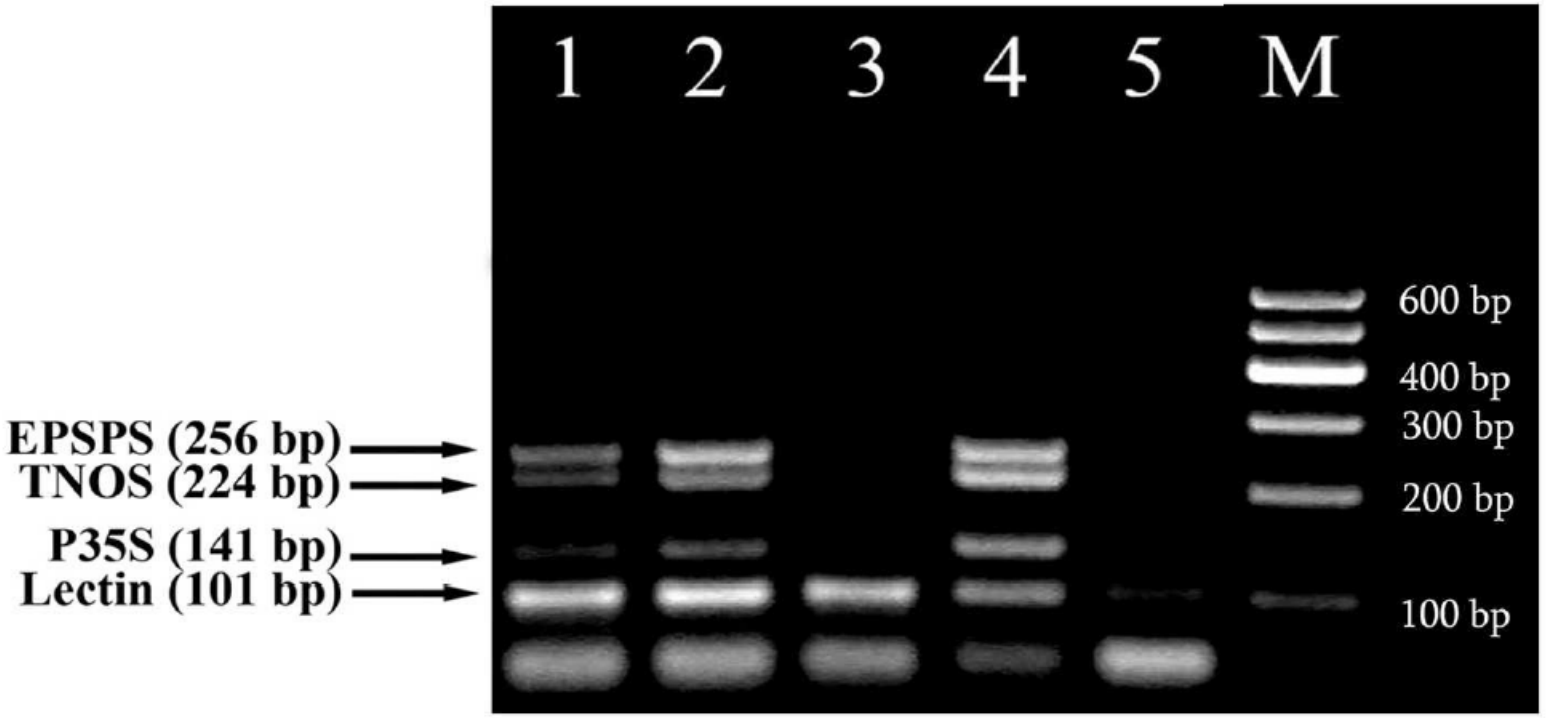
Agarose Gel Electrophoresis

- Prepare an agarose gel – melt agarose in buffer solution using heat, pour into mold with combs to cast well for DNA to be loaded
- Remove combs, submerge gel, load samples with loading/tracking dye, plus ladder
- Apply electrical current across gel to selectively size DNA through the “uniform” meshwork of agarose fibers. Small fragments move more quickly than larger ones. DNA is (-) charged so it migrates to the (+) electrode.

Agarose Gel Electrophoresis

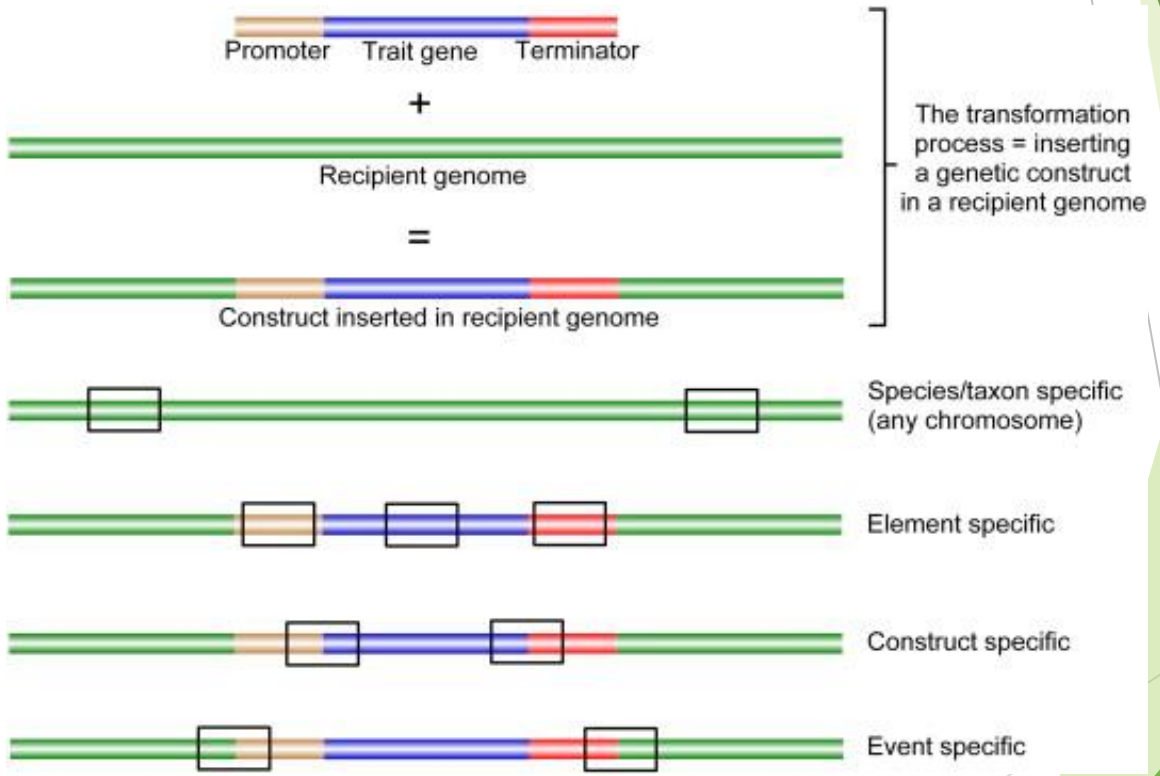
- After electrophoresis is complete, remove gel from rig, visualize with DNA binding stain like ethidium bromide, SYBR green/gold, etc. under a UV light





AP Seed Testing

- Many types of Adventitious Presence (AP) Testing
- Element specific, construct specific, event specific
  - Depends on the need of producer or consumer
- Element Specific could target promoter and terminator sequences associated with many GMO events
- Example: Conventional maize screened for p35S and tNOS sequence elements



AP Seed Testing

- Source of Element Specific PCR Assays from JRC; search JRC GMO Methods

Nr	ID	Title
1	QL-ELE-00-018	Qualitative PCR method for detection of nopaline synthase terminator (T-nos) (Barbau-Piednoir et al., 2014).
2	QL-ELE-00-024	Qualitative PCR method for detection of tE9 terminator (Debode et al., 2016).
3	QL-ELE-00-023	Qualitative PCR method for detection of T35S pCAMBIA sequences (Rischitor et al., 2016).
4	QL-ELE-00-029	Qualitative LAMP method for detection of CP4 epsps gene (Li et al., 2018).
5	QL-ELE-00-025	Qualitative duplex PCR method for detection of pat gene; bar gene (partim pat) (Debode et al., 2016).
6	QL-ELE-00-027	Qualitative LAMP method for detection of phosphinothricin N-acetyltransferase (bar) gene (Li et al., 2018).
7	QL-ELE-00-002	Qualitative PCR method for detection of Neomycin phosphotransferase II (ntpII) gene (ISO/FDIS 21569:2005).
8	QL-ELE-00-015	Qualitative PCR method for detection of Figwort Mosaic Virus 35S promoter
9	QL-ELE-00-010	Qualitative PCR method for detection of Figwort Mosaic Virus 35S promoter (Pan et al., 2007).
10	QL-ELE-00-019	Qualitative PCR method for detection of CP4 epsps gene (Barbau-Piednoir et al., 2014).
11	QL-ELE-00-003	Qualitative PCR method for detection of Neomycin phosphotransferase II (nptII) gene (BVL L 00.00-31, 1998)
12	QL-ELE-00-005	Qualitative PCR method for detection of Cauliflower Mosaic Virus 35S promoter (ISO/FDIS 21569:2005).
13	QL-ELE-00-021	Qualitative PCR method for detection of phosphinothricin N-acetyltransferase (pat) gene (Barbau-Piednoir et al., 2014).
14	QL-ELE-00-028	Qualitative LAMP method for detection of phosphinothricin N-acetyltransferase (pat) gene (Li et al., 2018).
15	QL-ELE-00-016	Qualitative PCR method for detection of cry1Ab/Ac gene

AP Seed Testing

- Construct Specific may detect a specific gene construct
- Example: cp4 gene (encodes EPSPS, confers glyphosate tolerance) present in NK603 or MON88017. This assay would not tell you which event is present however

AP Seed Testing

- Could be targeting a specific event
- Example: MIR604 assay will only detect MIR604, no other GMO event, or NK603 assays will only detect NK603, not MON88017
- Event detection needed when a country has not approved an event for import

AP Seed Testing

- Source of Event Specific PCR Assays from JRC; search JRC GMO Methods

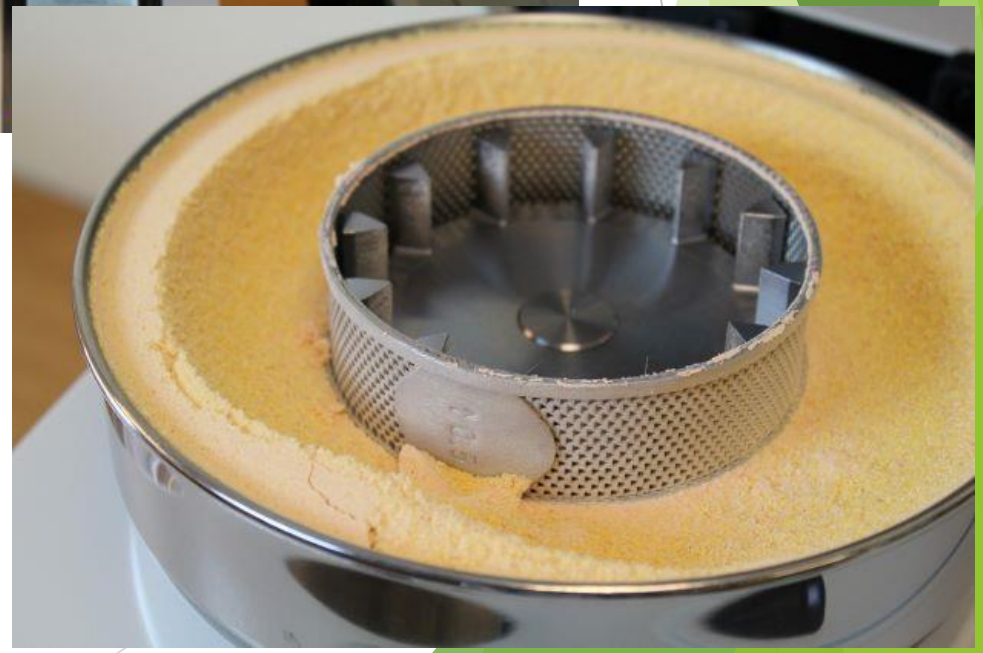
Results for query [id:QT-eve-zm*]		
Nr	ID	Title
1	QT-EVE-ZM-012	Quantitative PCR method for detection of maize event 59122 (Mazzara et al., 2006).
2	QT-EVE-ZM-025	Quantitative PCR method for detection of maize event MON 87403 (EURL GMFF, 2018).
3	QT-EVE-ZM-009	Quantitative PCR method for detection of maize event MON863 (Mazzara et al., 2005).
4	QT-EVE-ZM-003	Quantitative PCR method for detection of maize event MON87427(EURL GMFF, 2015).
5	QT-EVE-ZM-027	Quantitative PCR method for detection of maize event MZHG0JG (EURL GMFF, 2018).
6	QT-EVE-ZM-021	Quantitative PCR method for detection of maize event 98140 (Savini et al., 2011)
7	QT-EVE-ZM-005	Quantitative PCR method for detection of maize event MON87460 (Savini et al., 2011)
8	QT-EVE-ZM-002	Quantitative PCR method for detection of maize event 5307(EURL GMFF, 2014).
9	QT-EVE-ZM-008	Quantitative PCR method for detection of maize event NK603 (Mazzara et al., 2005).
10	QT-EVE-ZM-026	Quantitative PCR method for detection of maize event 4114 (EURL GMFF, 2018).
11	QT-EVE-ZM-014	Quantitative PCR method for detection of maize event GA21 (Charles Delobel et al., 2007).
12	QT-EVE-ZM-022	Quantitative PCR method for detection of maize event MIR162 (Charles Delobel et al., 2011)
13	QT-EVE-ZM-006	Quantitative PCR method for detection of maize event Bt11 (Mazzara et al., 2005).
14	QT-EVE-ZM-024	Quantitative PCR method for detection of maize event MON87411(EURL GMFF, 2016).
15	QT-EVE-ZM-020	Quantitative PCR method for detection of maize event MON810 (ISO/FDIS 21570:2005).

Overview of General AP Testing

- Obtain representative seed sample
- Determine what target(s) you need to assess: Event, element, protein?
  - Can also use ELISA, Western-blot, norther/southern blot for AP but not common
- Determine the approach or strategy: Quantitative or Semi-Quant?
- Get seed into extractable form: milling, crushing, harvest tissue?
- Extract DNA: magnetic bead, spin column via DNA binding membrane
- Quantify DNA: not always necessary
- Perform PCR: Real time or end point PCR
- Analyze Data: Visualize on agarose gel, process real time data, process end point fluorescence data, etc.

Seed Prep for AP PCR

- Most Common method is milling or grinding. Ideally want to produce a consistent particle size.
- Large # of seeds: ultra-centrifugal mill with sieve. Example Retsch ZM200 that utilizes a spinning rotor to shatter seed through a sieve which sizes the particles fairly evenly.

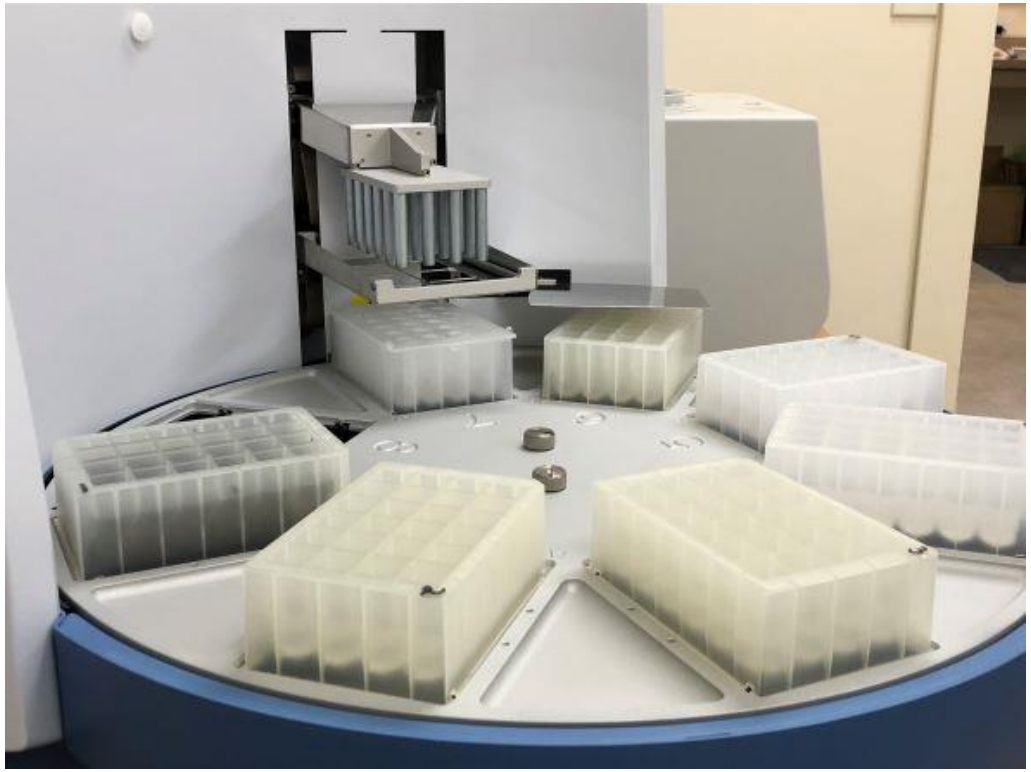


Seed/Leaf Prep for AP PCR

- If using small # of seeds per pool for Semi Quant PCR, then grinding in a coffee grinder, or other type of blender may be appropriate.
- Particle size can vary if pool size is say 100 seeds, so long as you can experimentally show that 1 in 100 seeds can be reliably detected.
- Same is true for leaf samples.
- Normally leaf samples are taken with a punch in a bulk fashion, then dried and homogenized with a Genogrinder.

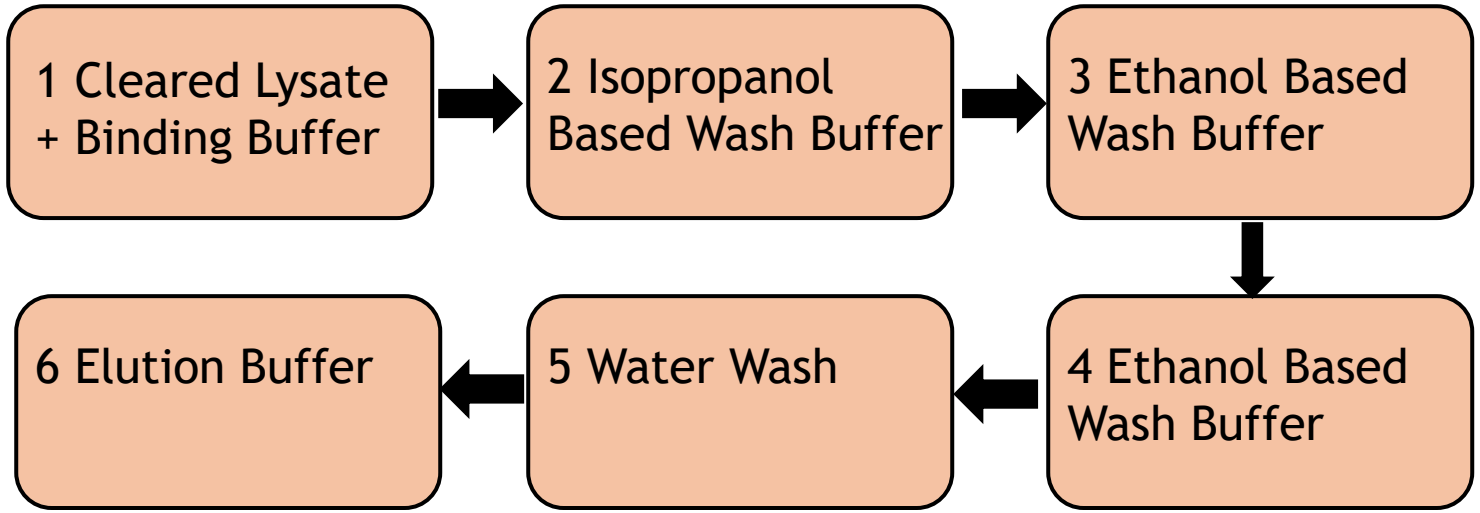
Sample Flour for DNA Extraction

- Obtain a representative flour sample from the milled seed.
- Mass of flour samples depends on mass of seed milled per seed pool
- Also depends on method or scale of DNA extraction kit or pipeline
- Large Scale extraction can be performed in multiple tubes or in a KingFisher 24 well plate



Lysis and precipitation

- Add a lysis buffer to flour and incubate at high temp (~70C), usually contains CTAB, sometimes proteinase
- Need to breakdown cell walls and begin to precipitate protein and sugars
- Next, precipitate DNA with ammonium/sodium acetate
- When DNA is bound to bead or membrane, begin wash step with alcohols.
- When DNA is clean, elute with water or buffer.
- Example of KingFisher 24 Well Extraction Steps, each bubble new plate that bound DNA is moved to

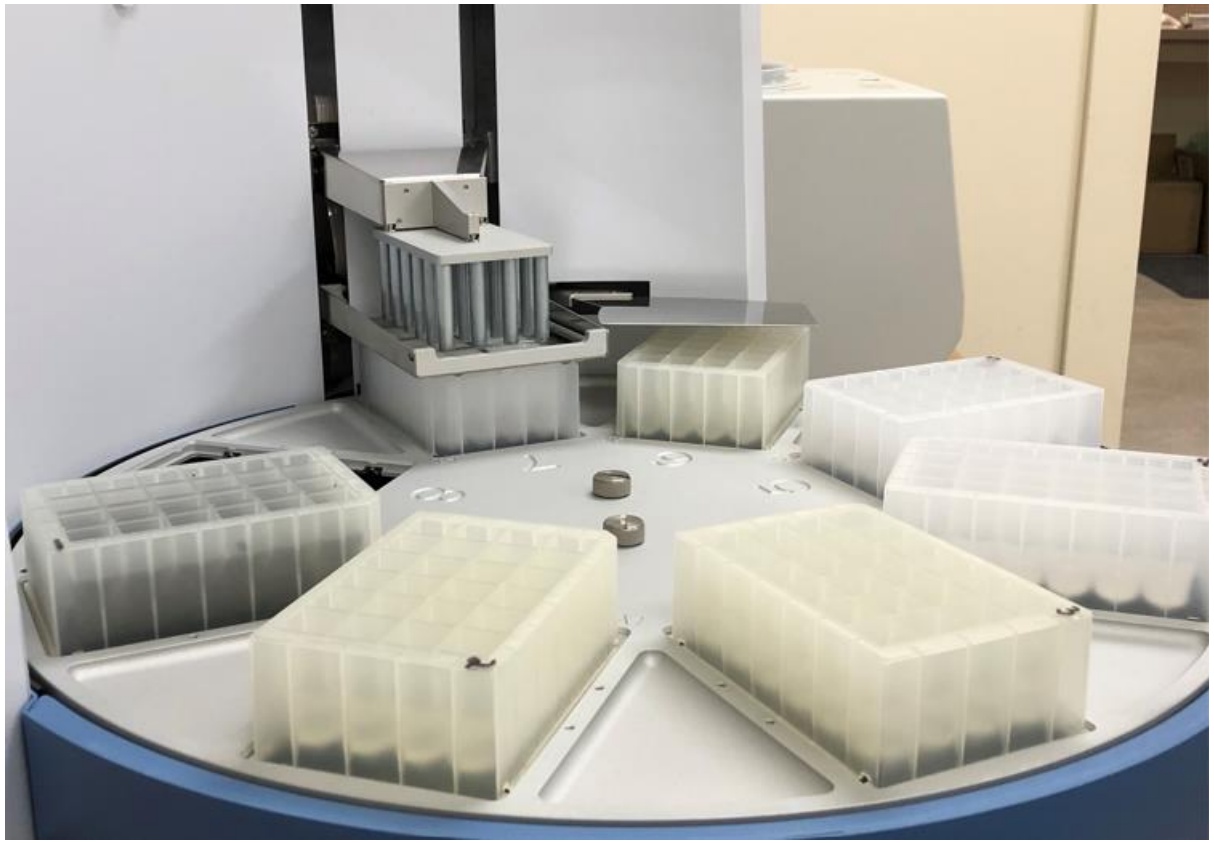


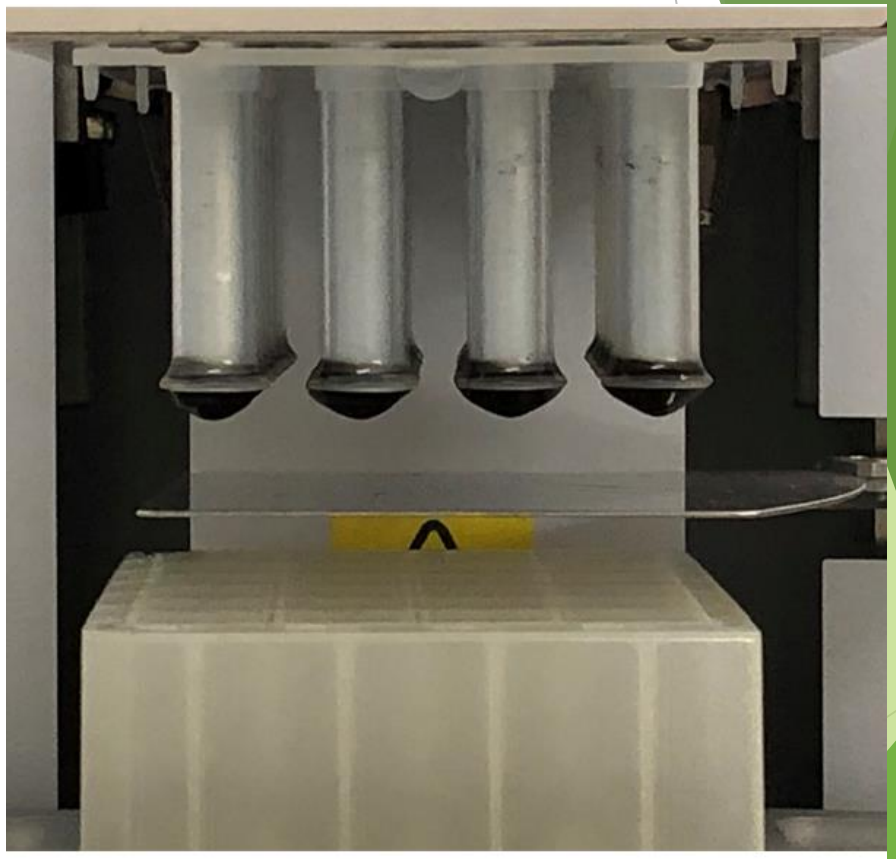
DNA Extraction

- Ideally, pure DNA is use for PCR because inhibitors can reduce the efficiency of the reactions by negatively affecting polymerase performance.
- Some PCR mixes can tolerate inhibitors, may be able to start with environmental sample
- Corn Leaf Punch DNA Extraction
  - Collect leaf punch
  - Homogenize/grind up tissue for efficient extraction
  - Add lysis buffer to break down cells to release DNA (Can stop here sometimes)
  - Purify DNA to remove polysaccharides, protein, oil, other debris
  - Alcohol-based wash step further purify DNA
  - DNA extraction kits available that use a DNA binding resin or membrane, OR magnetic beads to bind DNA.

DNA Extraction

DNA Extraction

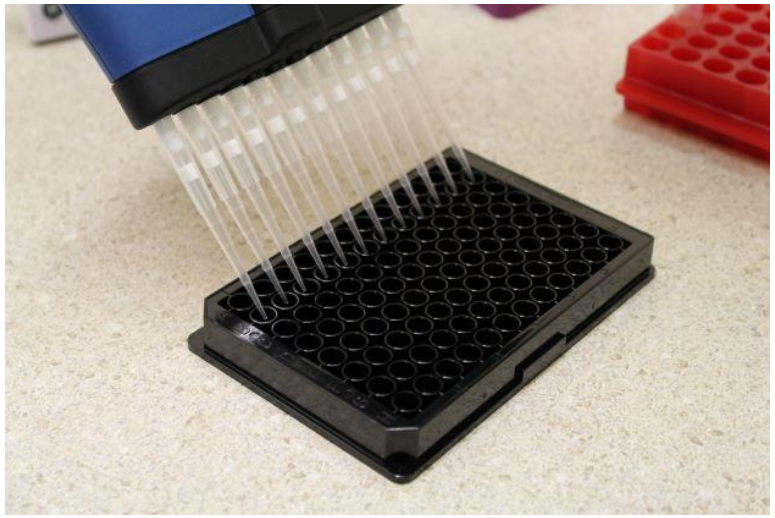




- Rods attract magnetic beads that bind DNA to facilitate movement of DNA through various wash buffers, and final elution

DNA Quantification and Normalization

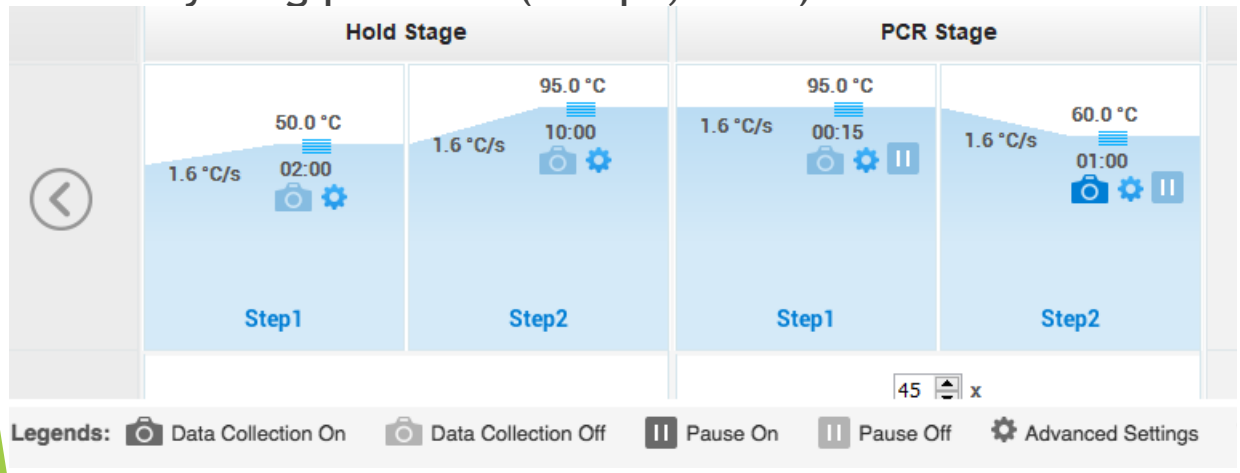
- Perform a PicoGreen DNA quantification assay
- Use a known set of DNA concentration standards to mix with DNA binding dye that fluoresces at certain wavelengths.
- Plot fluorescence values of unknown sample against standards to determine DNA concentration
- More Pipetting!! Wow, this time in a black plate!

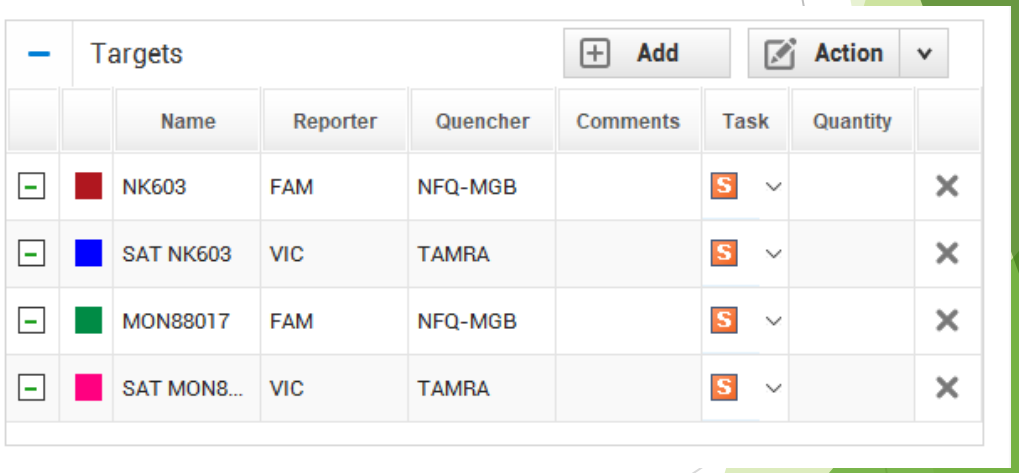


- Dilute DNA to 33ng/uL so that all unknows for PCR are at the same concentration

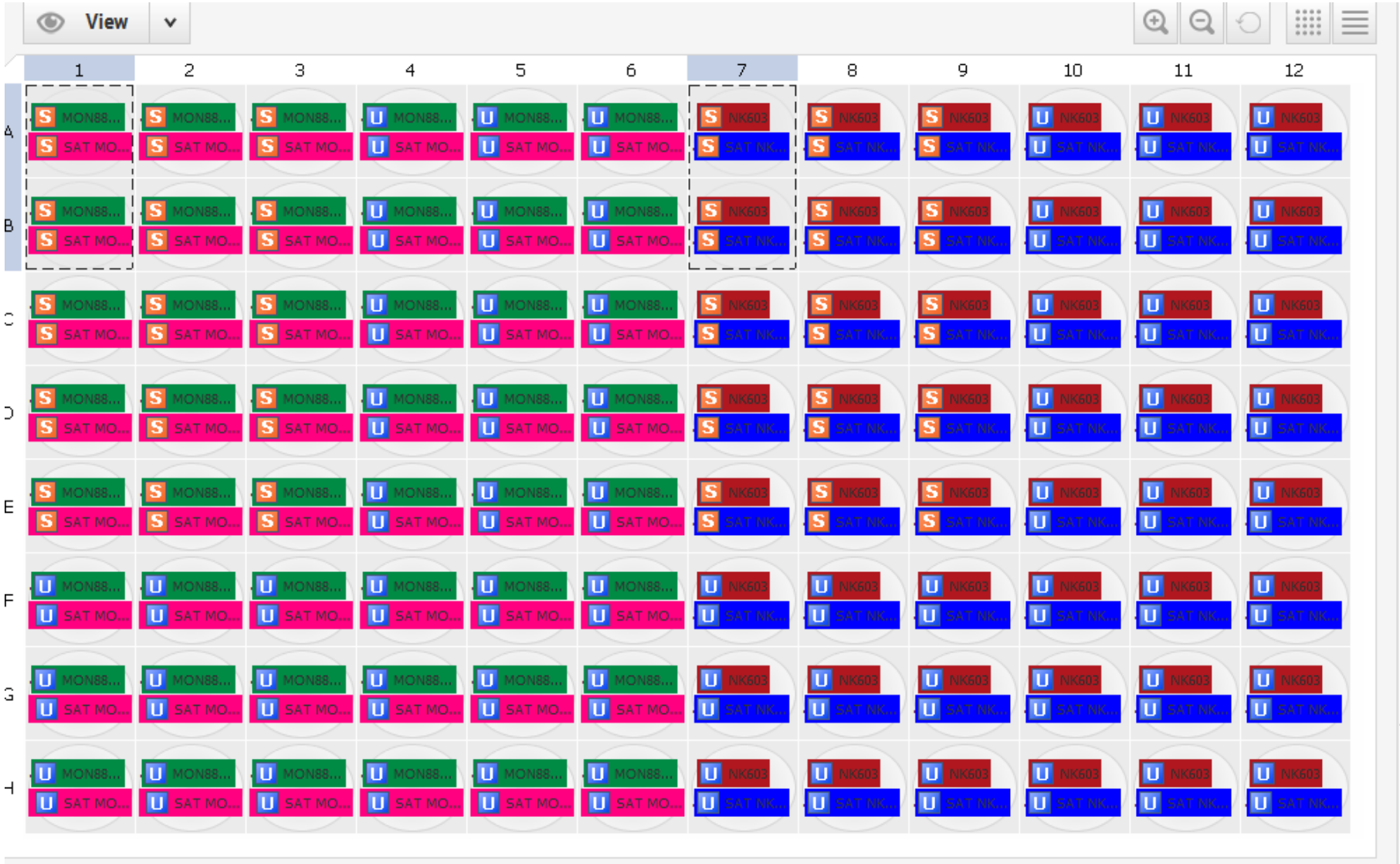
PCR Setup

- Obtain unknown DNA and standards or reference material DNA at correct concentration, PCR assay stocks (target and reference), add master mix (polymerase and buffers, cofactors)
- Pipet appropriate volumes in tubes or plates
- Place in Thermal cycler for 1hr 45 minutes roughly and use the appropriate cycling protocol (temps, time)





PCR Setup


View
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Real World Application in Seed Testing

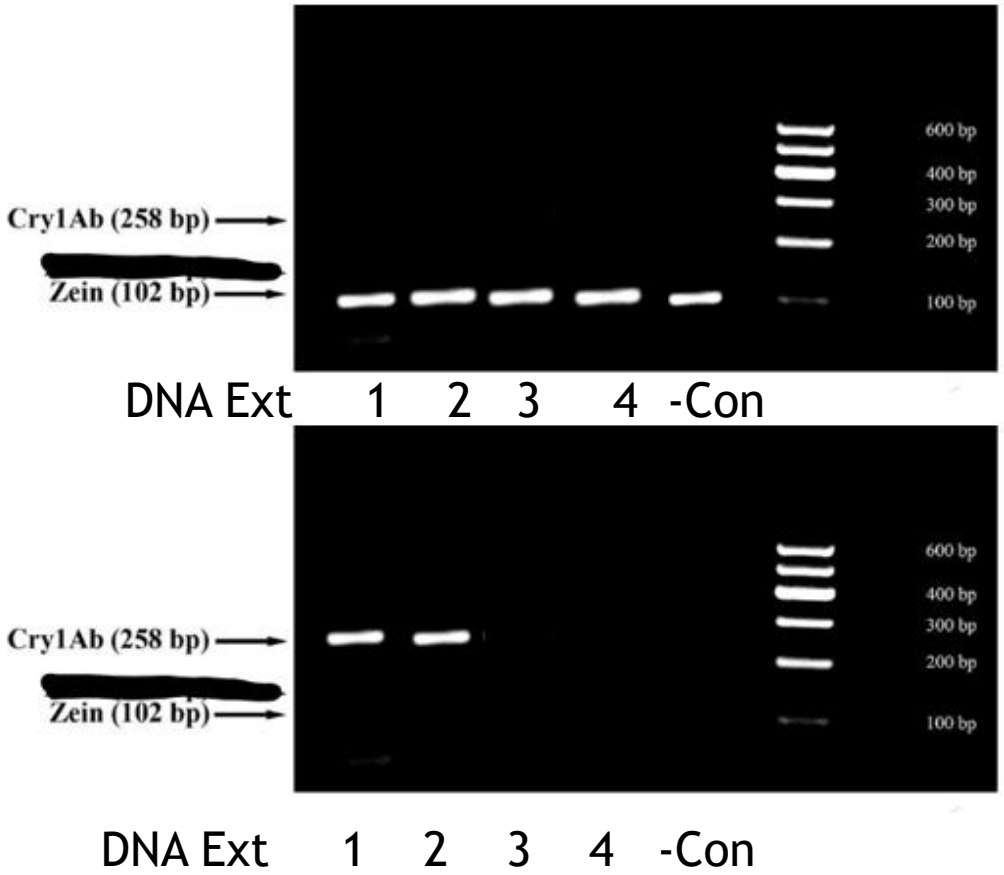
- Adventitious Presence of GMO Events in Seed
- Background
- MON89034 is DNA event containing DNA that encodes Cry2Ab2, a Bt protein that selectively kills lepidopteran insects
- Farmer Fred's seed company screens all conventional corn lots for the presence of Cry2Ab2 encoding DNA because many of their seed customers sell grain to food processors that have very low thresholds for the presence of Cry2Ab2 DNA. Farmer Fred's policy states "a seed lot showing presence of Cry2Ab2 is not acceptable for sale" to their conventional seed corn customers. Furthermore, any lot that shows presence of Cry2Ab2 DNA will be sold to Mike Stahr Farms for 30% of retail value.

Real World Application in Seed Testing

- 10lbs of seed is representatively sampled from a 500 bag lot of conventional corn.
- 500g of seed is taken from the 10lb rep. sample to be milled into a fine flour using an ultra-centrifugal mill.
- From the 500g of corn flour, 4X 0.8g flour sub samples are taken.
- A column-based DNA extraction is used purify DNA from the flour.
- The four DNA samples are placed into separate tubes with the components for PCR: Taq polymerase, dNTPS, Cry2Ab2 primers, cofactors. Run on thermal cycler for 45 cycles.
- Also run second set of reactions for a positive control (zein-encoding DNA). Zein a protein is present in all corn varieties.

Real World Application in Seed Testing

- After PCR, a small volume of the DNA from each tube is separately mixed with loading dye and loaded onto a 2% agarose gel. Gel is then visualizd under UV light.

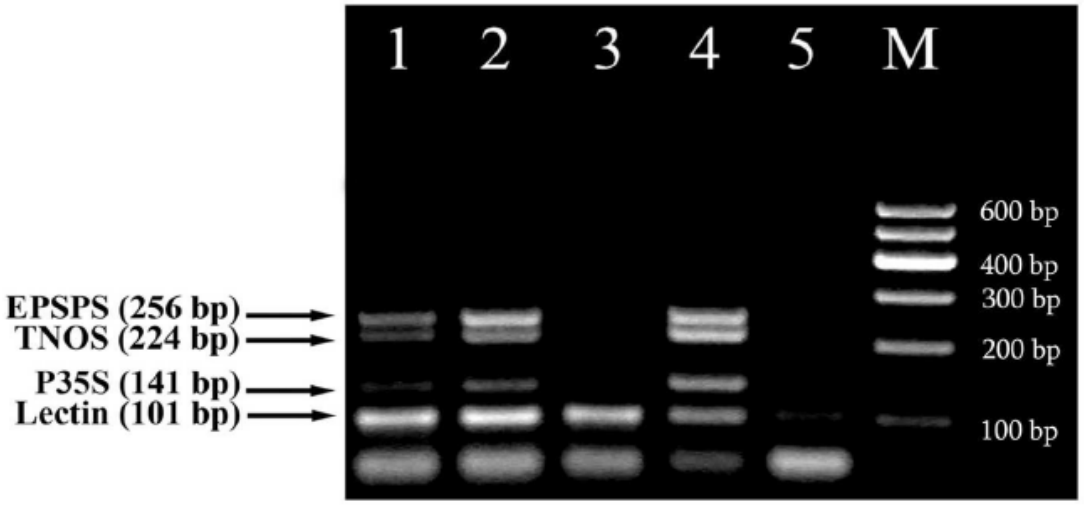


- Gel Results shown. What would the decision be for marketing this seed lot? Will Mike Stahr get discounted seed for 2022?
- What crucial component is missing in this seed testing layout?

Real World Application in Seed Testing

- More AP Testing: Farmer Fred's Seed Company sells conventional soybean seeds to certain customers to meet their non-GMO contract requirements. The company policy states: "All conventional soybean bin lots must be free of p35S, tNOS, and EPSPS DNA. Any bin lot showing presence of these genes must be sent to Mike Stahr Farms for feed use only."
- Lab protocol is to obtain 50lbs of representative seed sample, then divide it properly and draw 3 random samples of 454g each.
- Each 454g sample is then milled separately using an ultra-centrifugal mill. Flour samples of 1.0g are taken from each milled seed pool.
- DNA is extracted from the 1.0g flour samples using a KingFisher and magnetic bead extraction kit.
- DNA is quantified and concentration is adjusted to 33ng/uL for PCR
- PCR assays are prepared for multiplex PCR for p35S, tNOS, EPSPS, Lectin.
- After PCR, products are loaded onto a 2% agarose gel for visualization.

Real World Application in Seed Testing



- How many positive pools were observed?
- What is the status of this conventional soy seed lot?

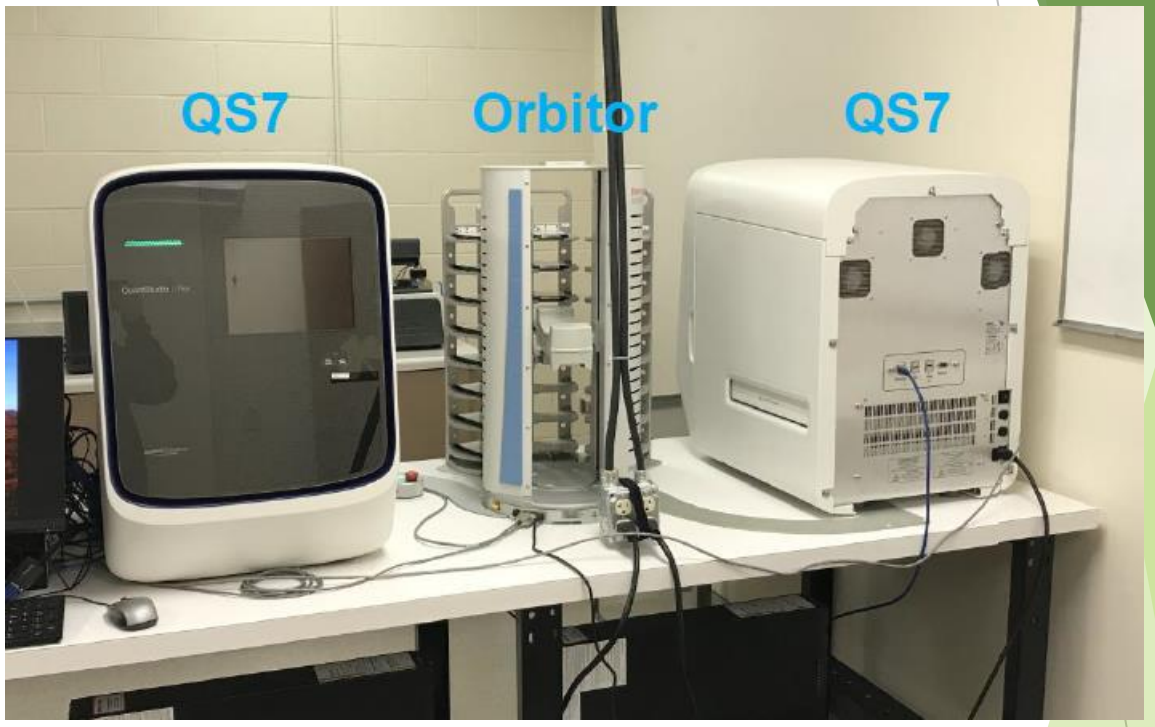
Real Time PCR / Quantitative PCR

- A real time thermal cycler ramps temperature but also has an onboard fluorometer to take a reading after each cycle.
- For purposes of this presentation, we will discuss the two most prominent types of real time PCR used in seed testing
- DNA binding chemistries (i.e. SYBRGreen, etc.)
  - dsDNA binding dye added to PCR Mix
- Probe Hydrolysis PCR (aka TaqMan PCR)
  - Dual-labeled Fluorescent Probe added to PCR Mix
  - Likely requires “retooling” of a normal PCR assay; primers need to be located to produce a product of ~75-150bp, with a DNA binding site in between for Probe

DNA Binding Chemistries

- Normal PCR with the inclusion of dsDNA binding dye
- Each cycle of PCR, 2X dsDNA is produced that dye can bind to
- 100X increase in fluorescence when bound
- Fluorometer fluorescence collects data each cycle upon extension step
- Quantification cycle (Cq) cycle is where fluorescence rises above threshold level.
- Standard Curves of gene on interest and endogenous gene of reference are prepared and run at same time as experimental samples
- Linear regression curve will generate y = mx + b
  - y = fluorescence value, m= slope of curve, x = Cq, and b = y intercept of curve
  - Determine the quantity of gene using formula: 10^((Cq-b)/m))

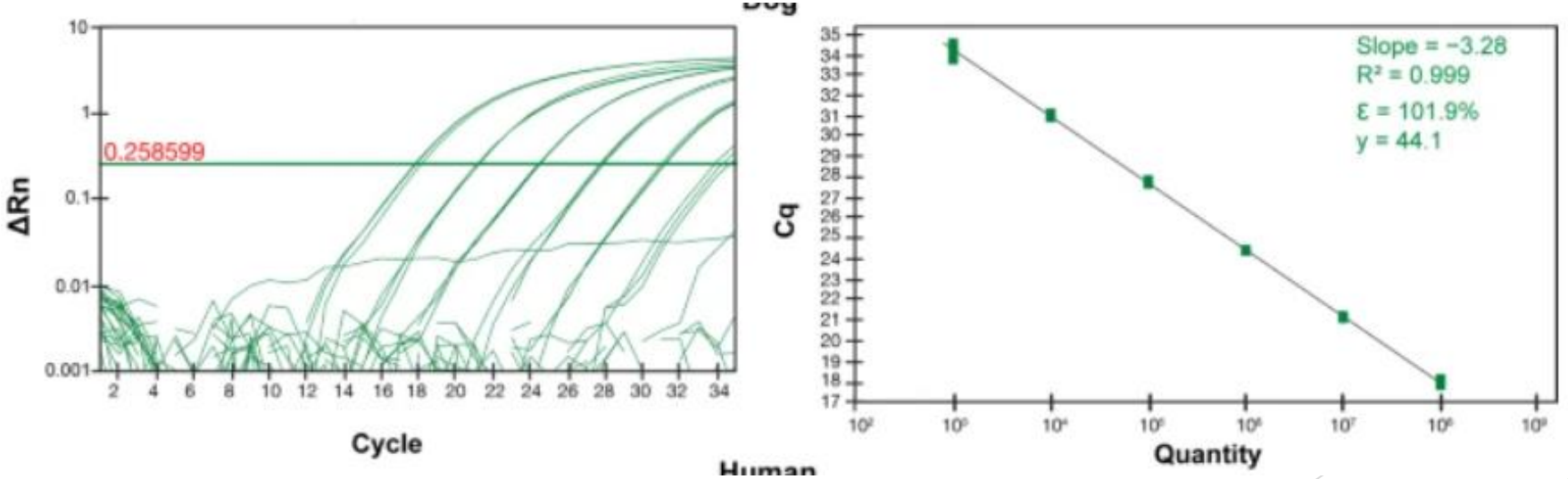
Real Time Thermal Cycler



- Real Time Cyclers come in all kinds of formats, in terms of wells or plate formats cycled; some have variable temperature rows or columns that are good for assay optimization.

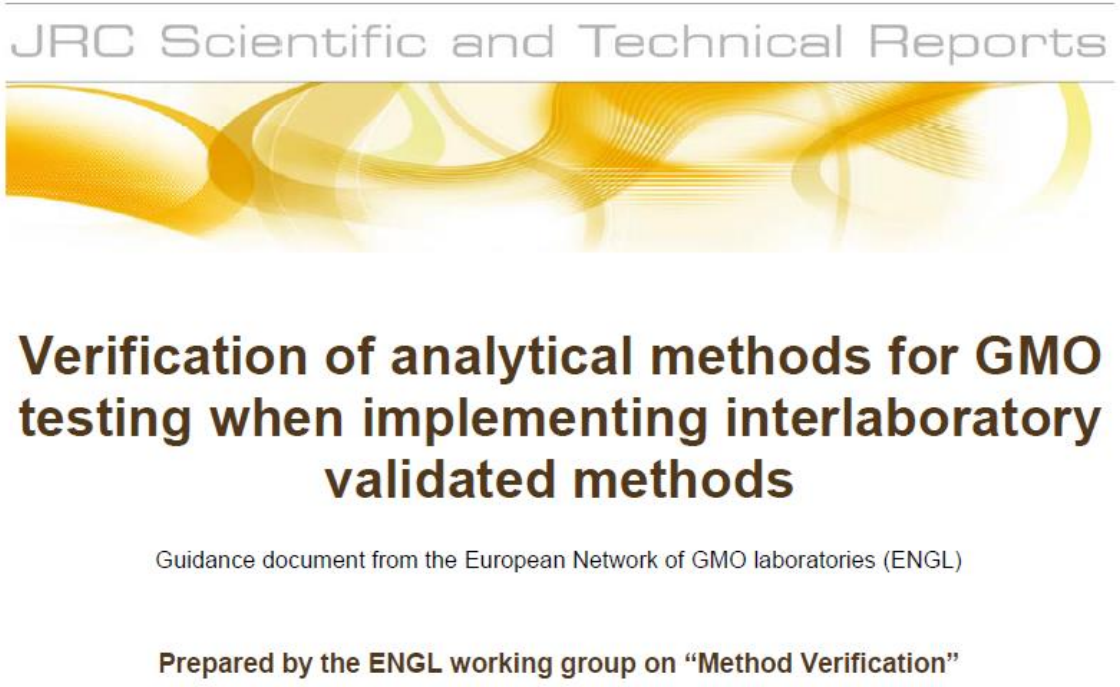
DNA Binding Chemistries

- Amplification Plot and Standard (Linear Regression) Curve
- Example below y is Y intercept (b) = 44.1, slope (m) = -3.28, therefore equation is y = -3.28x + 44.1
- Threshold is delta Rn = 0.258599
- Cq = Cycle of quantification, or cycle where threshold is reached = 20.4
- Concentration = 10^((Cq-b)/m)) = 10^((20.4-44.1)/-3.28)) = 5.89



Quantitative PCR terms

- Practical LOQ – Practical Limit of Quantification is the lowest relative quantity of target DNA that can be reliably quantified given a known number of target genome copies.
- Practical LOD – Practical Limit of Detection is the lowest relative quantity of target DNA that can be reliably (with >= 95% probability) detected, given a known number of target genome copies.



Probe Hydrolysis PCR

- Dual-labeled Fluorescent Probe added to PCR Mix
- Probe binds DNA in location between forward and reverse primer.
- Quencher inactivates fluorescence when in close proximity to reporter fluorophore, but cannot quench when probe is hydrolyzed.

Probe Hydrolysis PCR

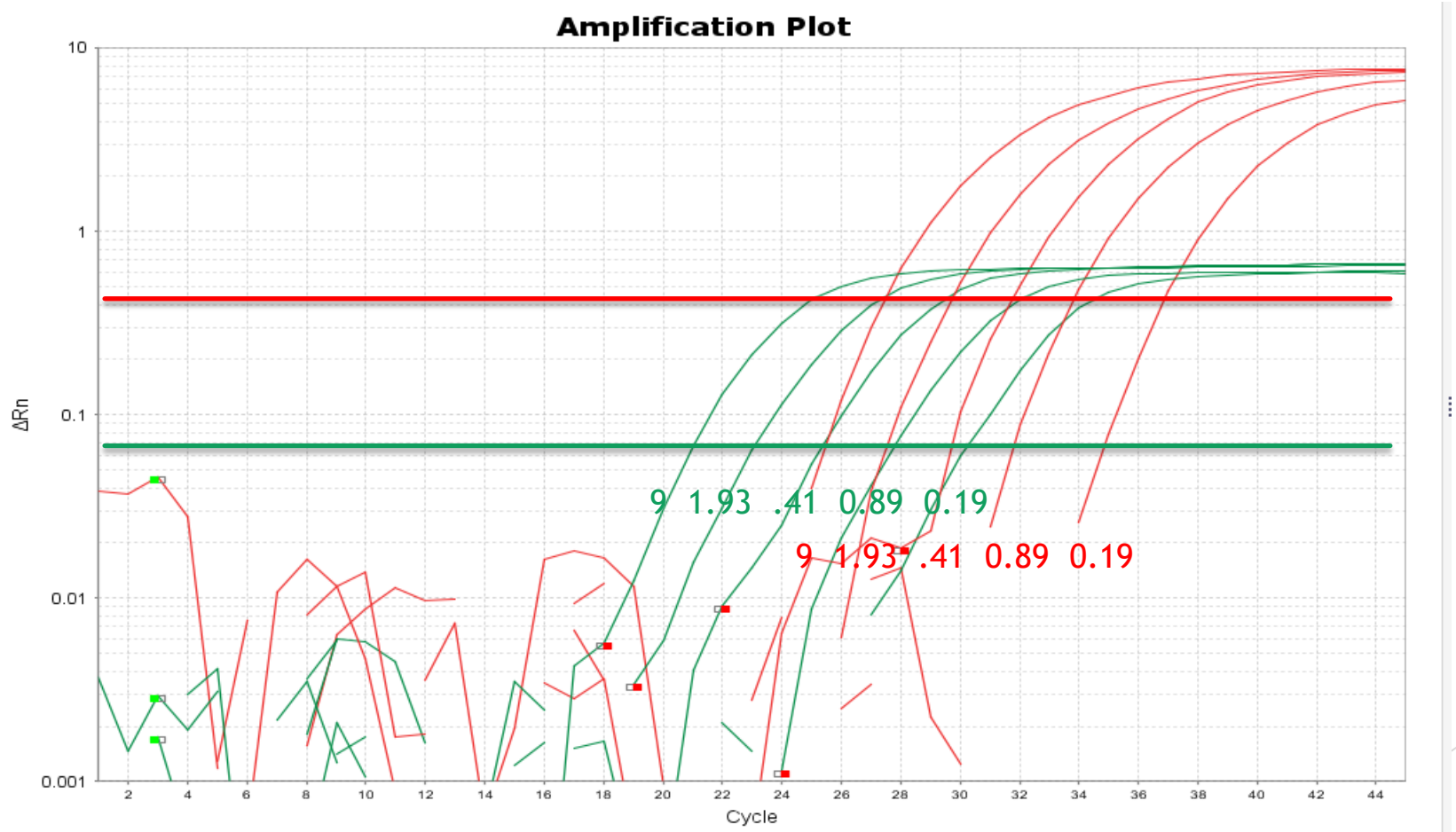
- Advantage: Very precise, in that any non-specific amplification will not be detected, compared to DNA binding chemistries. Lots of well documented, robust assays available for GMO testing using TaqMan assays.
- Disadvantage: must know complete sequence of region to be amplified to design probe. Reagents more expensive. PCR products can only be 75-150bp roughly.
- Example below of DAS59122-7 event (Herculex RW trait) Assay from JRC: https://gmo-crl.jrc.ec.europa.eu/gmomethods/

Features

Key	From	To	Length	Qualifier	Value
primer_bind	1	23	23	standard_name	Primer forward: DAS-59122-7-rb1f
				note	GGGATAAGCAAGTAAAAGCGCTC
				target	not specified
primer_bind	36	61	26	standard_name	RT-PCR probe: DAS-59122-7-rb1s
				note	FAM-TTTAAACTGAAGGCGGGAAACGACAA-TAMRA
primer_bind	63	86	24	standard_name	Primer reverse: DAS-59122-rb1r
				note	CCTTAATTCTCCGCTCATGATCAG
				target	not specified

Probe Hydrolysis Amplification Plot

- DAS-59122-7 Amplification Plot using FAM-TAMRA labeled event probe and VIC-TAMRA labeled adh1 endogenous reference gene probe, standards 9-0.019% DAS591227

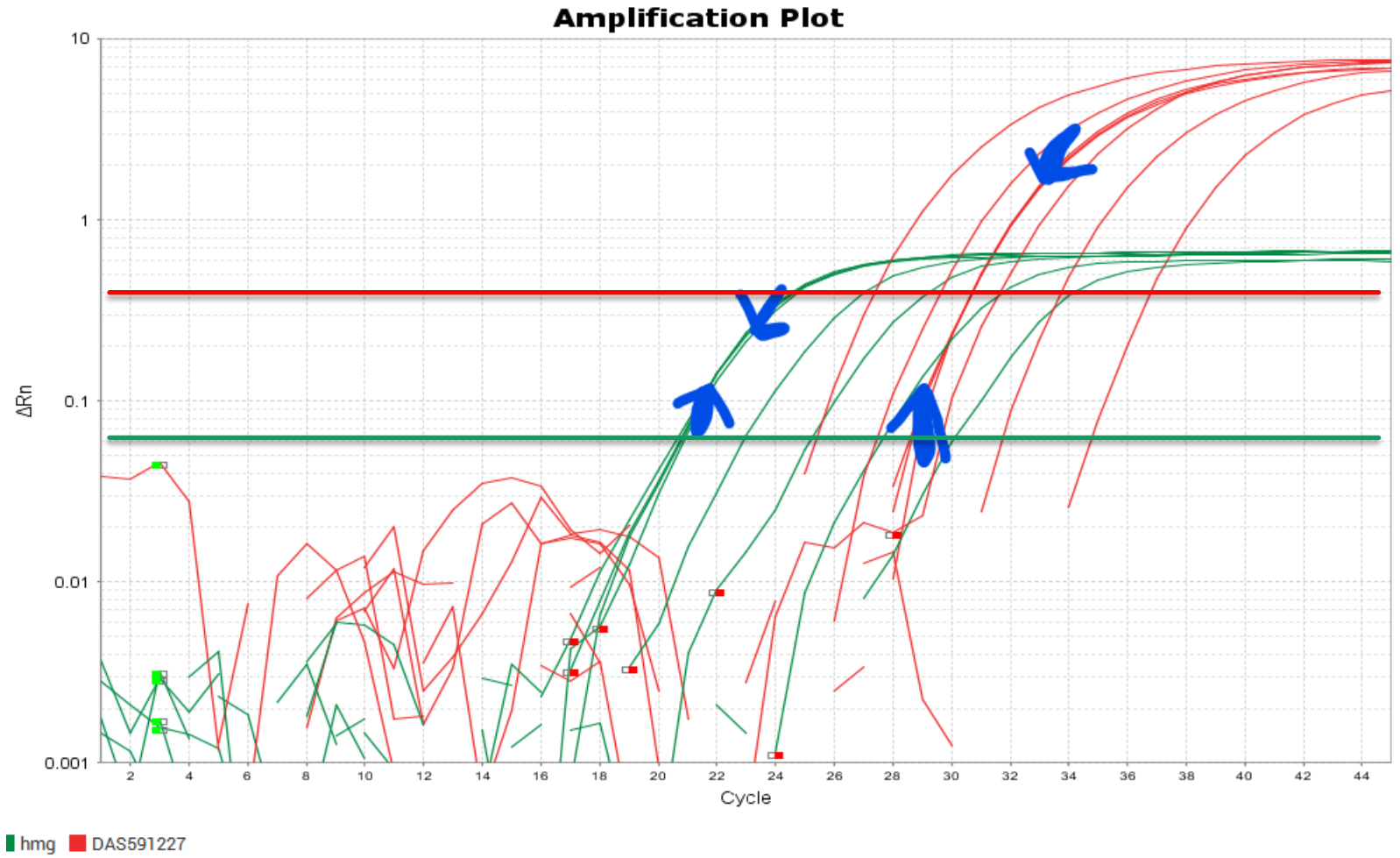


Red = DAS59122-7, Threshold = 0.400

Green = adh1, Threshold = 0.056

Probe Hydrolysis Amplification Plot

- Amplification shown for 1% DAS59122-7 CRM



Red = DAS59122-7,
Threshold = 0.400

Green = adh1,
Threshold = 0.056

Probe Hydrolysis Amplification Plot

- Data Calculations same as described for DNA binding chemistry.
- Can also use the Delta Delta Ct Method to determine the relative quantification using Ct data from a Calibrator (known concentration of target)
- Delta Ct = Ct (target) – Ct (ref gene)
- Delta Ct Delta Ct = Delta Ct (Unknown) – Delta Ct (Calibrator)
- Relative Quantification = 2^(-DeltaDeltaCt) = fold change compared to the calibrator
- Assumptions: equal primer efficiency, as well as ~90-110% PCR amplification efficiency in both target and reference genes, and reference gene expression is not affected by treatment OR reference gene is proportional in unknown and reference

Probe Hydrolysis Amplification Plot

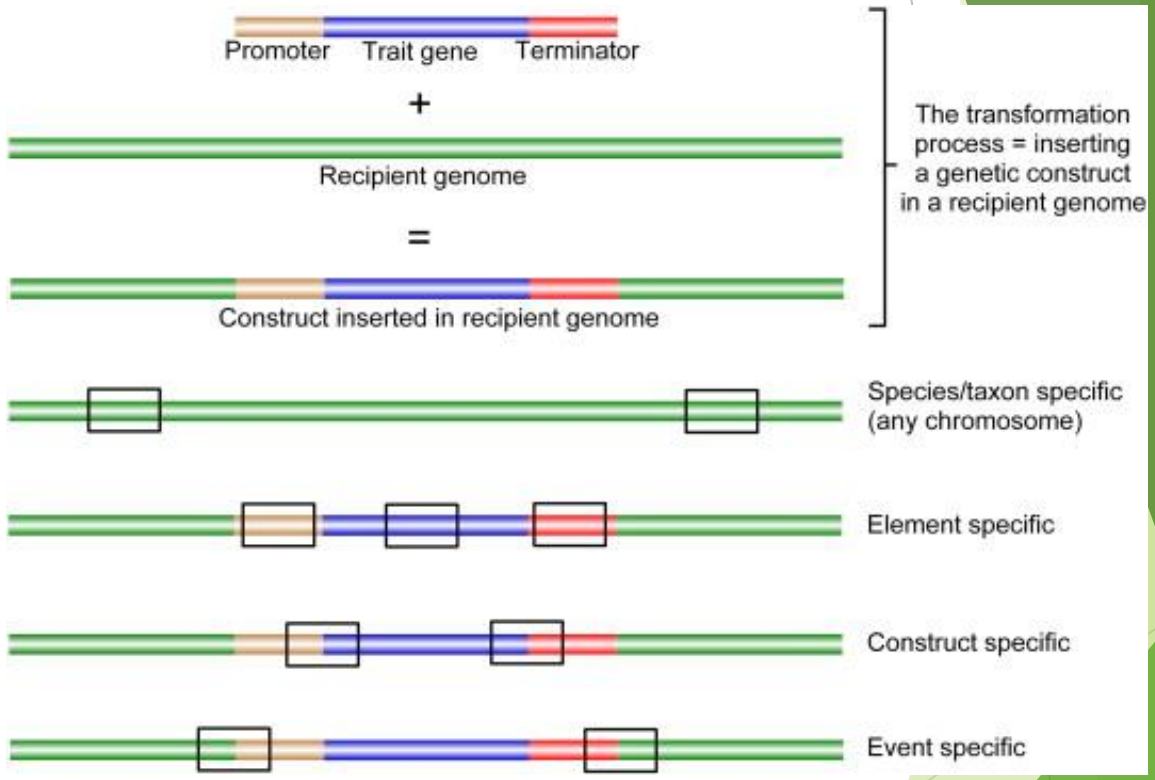
- Delta Ct = Ct (target) – Ct (ref gene)
- Delta Ct Delta Ct = Delta Ct (Unknown) – Delta Ct (Calibrator)
- Relative Quantification = 2^(-DeltaDeltaCt) = fold change compared to the calibrator
- Example Calculation:
- For Calibrator 1% CRM: DAS59122-7 Ct = 30.64 and adh1 Ct = 20.53
  - Calibrator Delta Ct = 30.64 – 20.53 = 10.11
- For Unknown: DAS59122-7 Ct = 30.91 and adh1 Ct = 20.23
  - Unknown Delta Ct = 30.91 – 20.23 = 10.68
- DeltaDeltaCt = 10.68-10.11 = 0.57
- Relative Quant = 2^(-0.57) = 0.673 So relative to 1% Calibrator, unknown is 0.673% DAS59122-7

Zygosity Testing for GMO Events

- Most zygosity PCR assays are probe-hydrolysis based
- Used to detect if an individual has one or two copies of the GMO event
- Hemizygous – one copy
- Homozygous – two copies
- Primers amplify DNA in the GMO event, probe binds region somewhere between probe binding sequences.

Zygosity Testing for GMO Events

- Primers amplify DNA in the GMO event, probe binds region somewhere between probe binding sequences.
- This could be element, construct, or event specific depending on the sequences of primers
- Normally a ddCt method is used for data analysis.
- Use a known homozygous sample as calibrator
- End result is determining if unknown is the same expression level as calibrator or roughly half that of calibrator
- Equal expression; unknown = homozygous. Half expression; unknown = hemizygous



Semi-Quantitative PCR

- Advantage over Quantitative Real Time PCR: Better at detecting the level of contamination in terms of individual seeds.
- This approach applies qualitative PCR assays to multiple small seed or leaf pools to generate a result based on statistical analysis
- Use SeedCalc8 program to evaluate qualitative data
- Example: Many GMO events contain multiple copies of promoters and terminators. Also, the zygosity level of the contaminant is not known. Contaminant could be hemizygous or homozygous.
- Example: (5) homozygous NK603 Seeds in 995 conventional seeds = 0.5% contaminating seeds. If you perform a real time PCR assay for tNOS, you may detect 10 copies of tNOS due to the contaminating seeds because homozygous NK603 seeds contain two copies each of tNOS leading you to believe that there are 1.0% contaminants. (5*2*2)= 20 / 1,000 = 2%
- If you performed a Semi Quant PCR of 20 pools of 50 seeds; you ,may find that 5 pools of the 20 are positive for NK603, and therefore the estimated contamination is 0.57%, NOT 2.0% like the real time result would suggest.

Events/Copies of Elements

- Zygosity levels and copies of elements influence real time PCR results

% Event CRM	Gene Standard	Zygosity	# Gene Copies	#p35S/Gene	Total #p35S	%p35S
0.5%	MON89034	Hemizygous	1	1	1	0.5%
0.5%	NK603	Homozygous	2	2	4	2.0%

Semi-Quantitative PCR

- Can use gel-based, DNA binding chemistries, Probe-based assays to generate qualitative result. Most labs use Probe-based.
- Must set a minimum value or measurement that constitutes a positive result
- Gel-based assay Example: Extract DNA from 25 seeds, perform PCR for NK603, any visible band at 183bp constitutes a positive result
- Probe-based Assay Example: Extract DNA from 150 seeds, perform PCR for NK603, any result of greater than 0.10% generated by deltadeltaCt using a 0.10% NK603 as a calibrator constitutes a positive result.

Semi-Quantitative PCR

- Visual representation of positive and negative pool results. + Pool = >0.1% NK603

150 Seed Pool	% NK603	Result
1	0.22	POSITIVE
2	0.06	NEGATIVE
3	0.03	NEGATIVE
4	0.02	NEGATIVE
5	0.02	NEGATIVE
6	0.16	POSITIVE
7	0.03	NEGATIVE
8	0.09	NEGATIVE
9	No Amp	NEGATIVE
10	0.08	NEGATIVE
11	0.22	POSITIVE
12	0.06	NEGATIVE
13	0.05	NEGATIVE
14	No Amp	NEGATIVE
15	No Amp	NEGATIVE

150 Seed Pool	% NK603	Result
16	0.02	POSITIVE
17	0.07	NEGATIVE
18	0.08	NEGATIVE
19	0.02	NEGATIVE
20	0.09	NEGATIVE
21	No Amp	NEGATIVE
22	0.03	NEGATIVE
23	0.09	NEGATIVE
24	0.09	NEGATIVE
25	0.08	NEGATIVE
26	0.32	POSITIVE
27	0.06	NEGATIVE
28	0.05	NEGATIVE
29	0.08	NEGATIVE
30	No Amp	NEGATIVE

#POSITIVE POOLS	5
#NEGATIVE POOLS	25
# POOLS TOTAL	30

Semi-Quantitative PCR

- Use SeedCalc8 to determine Estimated Contamination Levels

Impurity Estimation & Confidence Intervals (Assay measures impurity characteristic)

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

# of Seed Pools	30	Computed % in sample	0.12 %
# of Seeds per Pool	150		
Total Seeds Tested	4500		
		Measured property on seed pools	
# Deviants Pools	5		
		Desired Confidence Level	95 %

Upper Bound of True % Impurity	0.26		
	(95% confident that the lot impurity is below 0.26%.)		
2-sided CI for True % Impurity	*0.04*	to	*0.28*

Lower Bound of True % Purity	99.74		
	(95% confident that the lot purity is above 99.74%.)		
2-sided CI for True % Purity	*99.72*	to	*99.96*

Semi-Quantitative PCR

- Use SeedCalc8 to determine Estimated Contamination Levels

Impurity Estimation & Confidence Intervals (Assay measures impurity characteristic)

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

# of Seed Pools	30	Computed % in sample	1.79 %
# of Seeds per Pool	150		
Total Seeds Tested	4500		
# Deviants Pool	28	*Measured property on seed pools*	
		Desired Confidence Level	95 %

Upper Bound of True % Impurity	2.91		
	(95% confident that the lot impurity is below 2.91%.)		
2-sided CI for True % Impurity	*1.00*	*to*	*3.15*

Lower Bound of True % Purity	97.09		
	(95% confident that the lot purity is above 97.09%.)		
2-sided CI for True % Purity	*96.85*	*to*	*99.00*

Semi-Quantitative PCR

- Use SeedCalc8 to determine Estimated Contamination Levels

# of pools	seeds per pool	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
30	100	0	0.03	0.07	0.11	0.14	0.18	0.22	0.27	0.31	0.36	0.40	0.46	0.51	0.57	0.63	0.69	0.76	0.83	0.91	1.00	1.09	1.20	1.31	1.44	1.60	1.78	1.99	2.28	2.67	3.34	----

- Disadvantage of SemiQuant is that high levels of contamination are not able to be quantified.
- Example of pool strategy above: estimation of any contamination over 3.34% would not be possible. If you had an 8% contaminant seed mix, then likely there would be 30 positive pools, resulting in a “No Determination”.
- Real Time Quantitative PCR is normally limited by the highest concnetration of standard, for example 10% NK603 is highest, then that is the highest % of reliable quantification.

Trouble Shooting PCR

- Keep all tubes clearly labeled
- Develop recipes and reference when making assays stock or setting up reactions
- Repeatable, accurate pipetting
- Filter tips to reduce aerosolized DNA spread
- Keep reagents out of direct light whenever possible
- Use hot start Taq (almost all Taq for Real time PCR is)
- Always run multiple control/reference sample wells, preferably at more than one concentration
- Review PCR efficiency on ever PCR run to observe any upward or downward trends.
- DNA extraction efficiency- DNA quantification for every sample is good to see overall trends of DNA yields.