



PCR Results From Multiple HLB Testing Laboratories

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- Diagnostic laboratories 2005 (post discovery of HLB)
 - USDA APHIS
 - Beltsville, MD
 - Gastonia, NC
 - -FDACS
 - Gainesville, FL
 - -USDA-ARS
 - Ft. Pierce, FL
- Role of the laboratories
 - Mainly regulatory but did process some commercial samples



- Quickly became evident that a lab was needed for commercial samples
 - Southern Gardens Diagnostic Lab (10/2006)
 - Cooperative effort SG/UF/FCPRAC
 - -UF/IFAS-SWFREC (1/2008)
- Research Labs
 - Florida UF/USDA
 - Texas Texas A & M
 - California USDA



- As more labs came on line, growers were submitting samples to various labs
 - Some confusion based on interpretation of results (Ct)
 - Some confusion based on "split samples"
- Labs have different purposes
 - Routine testing
 - Regulatory
 - -Research



- Labs using various methods
- Interpretation of data and reporting of results varied
- Causing some confusion and some growers were starting to question the PCR testing process
- Laboratory Comparison

 Build confidence in the labs
 Identify and correct any problems



Why PCR?

- Causal organism can't be routinely cultured
- Can't use antibody-based testing (like used for CTV)
- No standard bioindexing test available (like used for viroids and psorosis)
 - Would be prohibitively expensive
- Hybridization assays were available but not as sensitive as PCR (radioactive reagents vs background....)
- PCR (polymerase chain reaction) is very specific and very sensitive
 - Relatively inexpensive
 - Transferable technology
 - High throughput
 - Considered as the "standard" test



Day Value \$0.01 1 **Basics of PCR** \$0.02 2 3 \$0.04 4 \$0.08 5 \$0.16 6 \$0.32 Would you rather have a check 7 \$0.64 8 \$1.28 for \$100,000 9 \$2.56 \$5.12 10 Or 11 \$10.24 12 \$20.48 A penny today, then that 13 \$40.96 14 \$81.92 amount doubled the next day, 15 \$163.84 16 \$327.68 and then that amount doubled 17 \$655.36 18 \$1.310.72 the next day and so on for 30 \$2.621.44 19 days? 20 \$5,242.88 21 \$10,485.76 22 \$20,971.52 23 \$41,943.04 24 \$83,886.08 PCR is essentially a penny a 25 \$167,772.16 26 \$335,544.32 day doubled but with DNA 27 \$671,088.64 28 \$1,342,177.28 29 \$2,684,354.56 7 30 \$5,368,709.12



Third cycle

Detection of Amplified Product

- Conventional PCR
 - Visual
 - Positive or Negative
- Real Time
 - Calculated
 - Continuous scale
 - Have to have a "cut off"
 - Ct value







Real Time PCR – Ct value



Comparison - Basics

- 13 laboratories (FL, MD, TX, CA)
- 276 Samples sent to all labs
 - Known positives
 - Known negatives
 - Unknowns
- DNA extracted by SGDL
 - Samples run "blind"
 - Some samples blind to all labs
- Diagnosis
 - Positive, negative or questionable
 - Ct values



Laboratories

- FDACS
 - Gainesville
 - Sun
 - Winter Haven
 - Sieburth
- Southern Gardens
 - Clewiston
 - Irey (2)
- Univ. of Florida
 - Lake Alfred
 - Brlansky
 - Rogers
 - Wang
 - Immokalee
 - Roberts

- USDA
 - Ft. Pierce
 - Hilf (2)
 - Shatters
 - Beltsville
 - Hartung (2)
 - Parlier
 - Hong Lin(2)
 - Riverside
 - Manjunath (2)
- Texas A & M
 - A & M Kingsville
 - DaGraça (1)



Testing Methodologies

- Conventional PCR
 - OI1/OI<mark>2c</mark> *
 - 2 labs
- Real Time PCR
 - Multiple primers
 - 16S
 - Li et. al. *
 - Other
 - B-Operon*
 - DNA Polymerase
 - Not described
- Multiple Regent Mixes
 - Optimized
 - Commercial master mixes
- Multiplex vs single Reaction

- Multiple PCR machines

 ABI
 - 7300
 - 7500
 - 7500 Fast
 - Cepheid Smart Cycler
 - Corbett Rotor Gene 6000
 - Stratagene MX3005P
 - Biorad IQ5
 - Biorad DNA Engine
 - MJ Research
- Multiple Detection Systems
 - Ethidium bromide
 - TaqMan
 - SYBR Green
 - EvaGreen



Consensus Diagnosis

_	_																
Primer			Wenbin primers	LAS LONG	HLBp Las (Li)	HLBas/COX	CQULA10/COX	Wenbin Li primers with P	Wenbin Li prime	r Weben Li	BoveColetta-Filho	OI1/OI2c	HLBas, HLBr, H	IL HLBas, HLBr, H	Li primers	Li primers	CQULA04F-CQUI
PCR Type	les les		Real Time	Real Time	Real Time	Real Time	Real Time	Real Time	Real Time	Real Time	Conventional	Conventional	Real Time	Real Time	Real Time	Real Time	Real Time
Detection	l l	s s	Taqman	Other	Taqman	Other	Other	Taqman	Taqman	Taqman	ETBr	ETBr	Taqman	Taqman	Taqman	Taqman	Other
Machine	Ţ	cens	Cephied Smart	C Rotor Gene 6000	AB7500	SMART CYCLE	R SMART CYCLER	R Stratagene Mx3005P	Stratagene Mx30	Applied Biosy	sterBio-Rad DNA engine	MJPTC200	Applied Biosyst	terApplied Biosyste	ei ABI 7500	ABI 7500	Applied Biosyster
Sample Number	Υ ^μ	Diac		1 2	3	6	7	11	12	16	17	21	26	27	31	32	33
CG08012		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08013		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08014		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08015		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08016		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08017		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08018		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08019		-	-	-	-	-	-	?	-	-	-	-	-	-	-	-	-
CG08020		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08021	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
CG08022	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08023	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08024	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08025	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CG08026	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08027	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08028	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
CG08029	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08030	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08031	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08032	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08033	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+
CG08034	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08035	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08036	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08037	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08038	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08039	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
CG08040	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+
CG08041	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08042	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08043	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08044	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CG08045	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Summary of Results - Diagnosis



- False Positive (+ when the sample is negative)
 - Could have severe implications for nurseries, budwood suppliers
- False Negative (- when the sample is positive)
 - Could have severe implications for regulatory samples



Implications of False + and False -

- False Positive (+ when the sample is negative)
 - Could have severe implications for nurseries, budwood suppliers
- Could be OK if running regulatory and research samples (want to err on the side of finding positives, most likely would be re-run)

- False Negative (- when the sample is positive)
 - Could have severe implications for regulatory samples
- Could be OK if running commercial samples (likely not to be regulatory implications)



Bottom Line for Florida Labs

Primers	Li	Li	Li	OI1/OI2c	Li	Li	Li	DNA Polymerase
Purpose	Industry							
Regulatory	Yes		-	Yes				
Known Negatives	100%	100%	98%	100%	100%	99%	100%	100%
Known Positives	100%	100%	98%	97%	97%	97%	100%	100%
All Negatives	99%	97%	97%	100%	99%	98%	100%	100%
All Positives	100%	100%	99%	97%	97%	97%	100%	100%
Questionables	71%	100%	86%	0%	86%	86%	29%	29%
Туре	RT	RT	RT	Conv	RT	RT	RT	RT

- All Florida labs (and CA lab running psyllids) running industry samples are doing a good job and are producing similar results
- Differences how conservative they are



Summary of Results - Diagnosis

1	ТХ	FL		CA/ FL	CA/ FL		FL	FL			FL	
Primers	Li	Li	Li	Li	Li	Li	Li	Li	Li	Li	Li	Li
Purpose	Industry	Industry	Research	Industry	Industry	Research	Industry	Industry	Research	Research	Industry	Research
Regulatory	Yes	Yes										
Known Negatives	100%	100%	100%	100%	98%	100%	100%	99%	100%	100%	100%	99%
Known Positives	97%	100%	69%	100%	98%	100%	97%	97%	67%	78%	100%	98%
All Negatives	100%	99%	100%	97%	97%	100%	99%	98%	100%	100%	100%	99%
All Positives	97%	100%	69%	100%	99%	100%	97%	97%	67%	78%	100%	99%
Questionables	0%	71%	0%	100%	86%	43%	86%	86%	0%	0%	29%	0%

Primers	β-Operon	β-Operon
Purpose	Research	Research
Regulatory		
Known Negatives	100%	99%
Known Positives	5%	94%
All Negatives	100%	100%
All Positives	4%	94%
Questionables	0%	0%

Primers	16S Other	DNA Polymerase	Not Specified		
Purpose	Research	Industry	Research		
Regulatory					
Known Negatives	96%	100%	100%		
Known Positives	98%	100%	98%		
All Negatives	91%	100%	100%		
All Positives	99%	100%	99%		
Questionables	0%	29%	0%		

		1 6
Primers	011/012c	011/012c
Purpose	Research	Industry
Regulatory		Yes
Known Negatives	100%	100%
Known Positives	72%	97%
All Negatives	100%	100%
All Positives	73%	97%
Questionables	0%	0%



Lab Report

HLB Tes	ting Rep	ort			Fax: Email		ssay Date Range: (11/2007 to 4/11/2007				
USSC/Southern	ı Gardens Diagn	ostic Lab	1	Cou	nty						
Diagnosis	HLB Positi	ive									
Block	Row	Tree	СТ	Local Id:	Lab Id	Collected	Received	Assayed	ed Sample submitted by:		
	5	70	22.50	03-29-63	7741	3/27/2007	4/4/2007	4/11/2007			
	8	56	25.36	03-29-63	7746	3/27/2007	4/4/2007	4/11/2007			
	8	58	23.14	03-26-07	7745	3/27/2007	4/4/2007	4/11/2007			
	11	71	26.66	03-29-63	7742	3/27/2007	4/4/2007	4/11/2007			
Diagnosis	11 71 No HLB Found										
Block	Row	Tree	СТ	Local Id:	Lab Id	Collected	Received	Assayed	Sample submitted by:		
	1	82	33.83	03-09-29	7743	3/27/2007	4/4/2007	4/11/2007			
	12	74	39.87	03-29-24	7740	3/27/2007	4/4/2007	4/11/2007			
Diagnosis	agnosis Question										
Block	Row	Tree	СТ	Local Id:	Lab Id	Collected	Received	Assayed	Sample submitted by:		
	3	19	31.33	03-29-37	7744	3/27/2007	4/4/2007	4/11/2007			
Number Po	sitive 4	Num	per Que	tionable 1	Total sa	mples 7	Total Posi	sitive (+ and questionable) 71.4%			

CT< 30 Positive CT between 30-32 Questionable CT>32 No HLB Found





Threshold Determination – Depends on Purpose

Distribution of CT values





Ct Values – Li (different labs)





PORATION

Distribution of Ct Values – Known Negatives

1		10a		10b		3		5 rep 1		5 Rep 2	
Bin	Frequency	Bin	Frequency	Bin	Frequency	Bin	Frequency	Bin	Frequency	Bin	Frequency
29	134	29	134	29	134	29	134	29	134	29	134
30	0	30	0	30	0	30	0	30	0	30	0
31	0	31	0	31	0	31	0	31	0	31	0
32	0	32	0	32	0	32	0	32	0	32	0
33	0	33	0	33	1	33	0	33	0	33	0
34	0	34	0	34	1	34	0	34	0	34	0
35	0	35	0	35	1	35	0	35	0	35	0
36	2	36	1	36	6	36	1	36	0	36	1
37	7	37	10	37	22	37	1	37	1	37	3
38	10	38	15	38	30	38	8	38	0	38	6
39	9	39	15	39	23	39	10	39	8	39	9
40	114	40	101	40	58	40	122	40	133	40	123
More	0	More	0	More	0	More	0	More	0	More	0

6		7		8		9a		9b		
Bin	Frequency	Bin	Frequency	Bin	Frequency	Bin	Frequency	Bin	Frequency	
29	134	29	134	29	135	29	134	29	134	-
30	0	30	0	30	0	30	0	30	0	•
31	0	31	0	31	0	31	0	31	0	
32	0	32	0	32	0	32	0	32	0	n
33	0	33	0	33	0	33	0	33	0	••
34	0	34	1	34	1	34	1	34	0	h
35	0	35	0	35	0	35	1	35	1	D
36	0	36	0	36	1	36	8	36	0	\sim
37	0	37	0	37	0	37	6	37	4	e
38	0	38	1	38	4	38	18	38	9	
39	0	39	0	39	18	39	16	39	13	6
40	142	40	140	40	117	40	92	40	115	
ore	0	More	0	More	0	More	0	More	0	

•Threshold needs to be set for each lab/assay





Sending in samples to the lab.....







Reasons to Send in Samples

- Confirm the presence or absence of HLB
- Train scouts
- Quality control for scouting crews
- Research
 - Private
 - University
 - Federal
- Nurseries



Number of Samples Received By SGDL



Number of Samples Received Per Month at SGDL

Through Oct 2008 n=79,429 (~another 11,000 received and in process)



Lab Turn-Around Time

- Current turn around time 6-12 weeks depending on the lab and month
- Not acceptable
 - -Will be hiring more people
 - -Educate growers



Issues

- Some growers requiring a test for every tree removed THE LABS CAN'T HANDLE THIS
 - Expensive
 - Increases turn-around time
- Use the labs to train and QC the scouts TRUST YOUR SCOUTS
 - Many scouting crews 95+% in agreement with lab testing



Industry Labs

Valuable resources

Please use them responsibly



Thank you for your attention!

Questions?