

Update on Method Development of USDA APHIS PPQ Diagnostic Tests for *Phytophthora ramorum*

Center for Plant Health Science and Technology

**National Plant Germplasm and Biotechnology
Laboratory - Beltsville**

U.S. Lab Capacity for *P. ramorum*

NPPLAP (originally Provisional Approval program)
-diagnostic determinations for USDA regulatory purposes

25 participating labs (in *P. ramorum* Program)

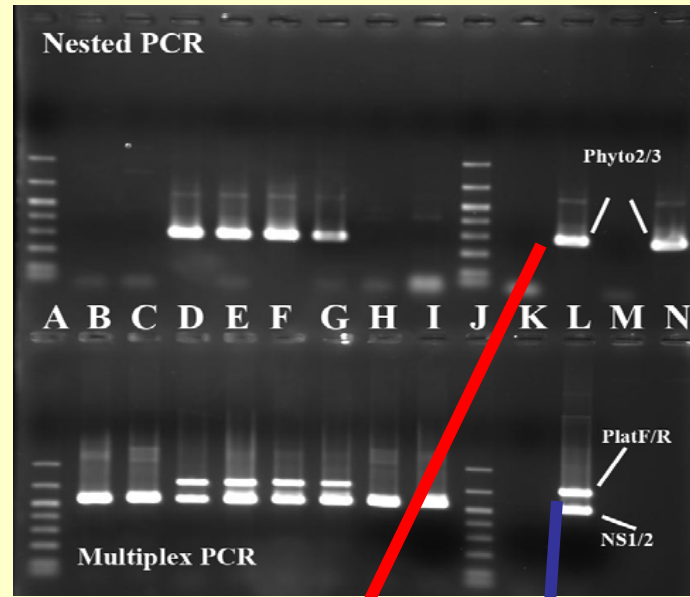
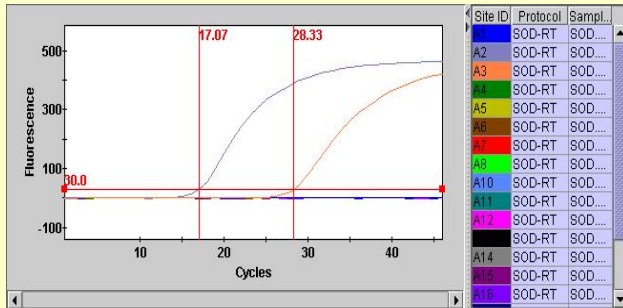
- State Department of Agriculture
- National Plant Diagnostic Network
- Agricultural Experimentation Stations
- other USDA labs

- USDA Regulatory Labs

History: Molecular Diagnostics of *P. ramorum*

1. Nested PCR based on ITS (with multiplex PCR for *P. lateralis* and NS product for DNA QC) - 2003
2. Real-time PCR based on ITS (multiplexed with plant Cox for DNA QC) - 2006
3. Evaluation of Real-time PCR protocols that have been developed since beginning of Emergency Program

Molecular Target Selection

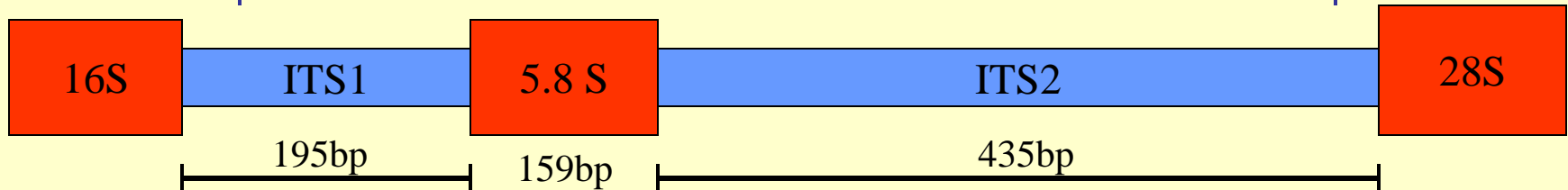


CSL-Real-time Product

Phyto2 / Phyto3 Product (2nd Rd. Nested)

Phyto1 / Phyto4 Product (1st Rd. Nested)

PlatF / PlatR Product



Objectives:

Improve current protocols based on recommendations of *P. ramorum* Science Panel (June, 2004), NPB/PPQ Program Review (July, 2004) and other stakeholder recommendations

- Utilize High-Throughput Diagnostic Platform

- Use Multiple Genomic Targets

- Higher specificity/lower false positive rate

 - Control reactions for high degree of Quality Assurance

Real-time PCR (vs. conventional PCR):

- **Amplicon is visualised as the amplification progresses.**
- Closed system
 - No post-amplification processing
 - *Rapid*
 - *Reduced contamination*
 - *Automation/high throughput/Cost effective*
- More tests /less reagents/standardized cycling conditions
- Increased sensitivity/specificity

Disadvantages of real time PCR:

- Risk of false negative reactions (due to miss-matches).
- Number of amplicons detected is limited by the number of fluorophores.
- Expensive to start up

Table 1: Alternative Real-Time PCR Diagnostics for *P. ramorum* Assays Currently Under Evaluation:

Gene	Assay	Developer	Status
<i>Ypt1</i>	TaqMan [®]	CSL ^a	Preliminary Validation Completed
ξ-Tubulin	TaqMan [®]	CFIA/CFS ^b	Preliminary Validation Completed
ITS	TaqMan [®]	CFIA/CFS ^b	Preliminary Validation Completed
Elicitin	TaqMan [®]	CFIA/CFS ^b	Preliminary Validation Completed
<i>Cox</i>	TaqMan [®]	ARS-Salinas ^c	Preliminary Validation Completed

^a Schena, Hughes & Cooke, 2006, *Molecular Plant Pathology* 7:365-379

^b Bilodeau, L'Vesque, de Cock, Duschaine, Briere, Uribe, Martin & Hamelin. 2007 *Phytopathology* 97 632-642.

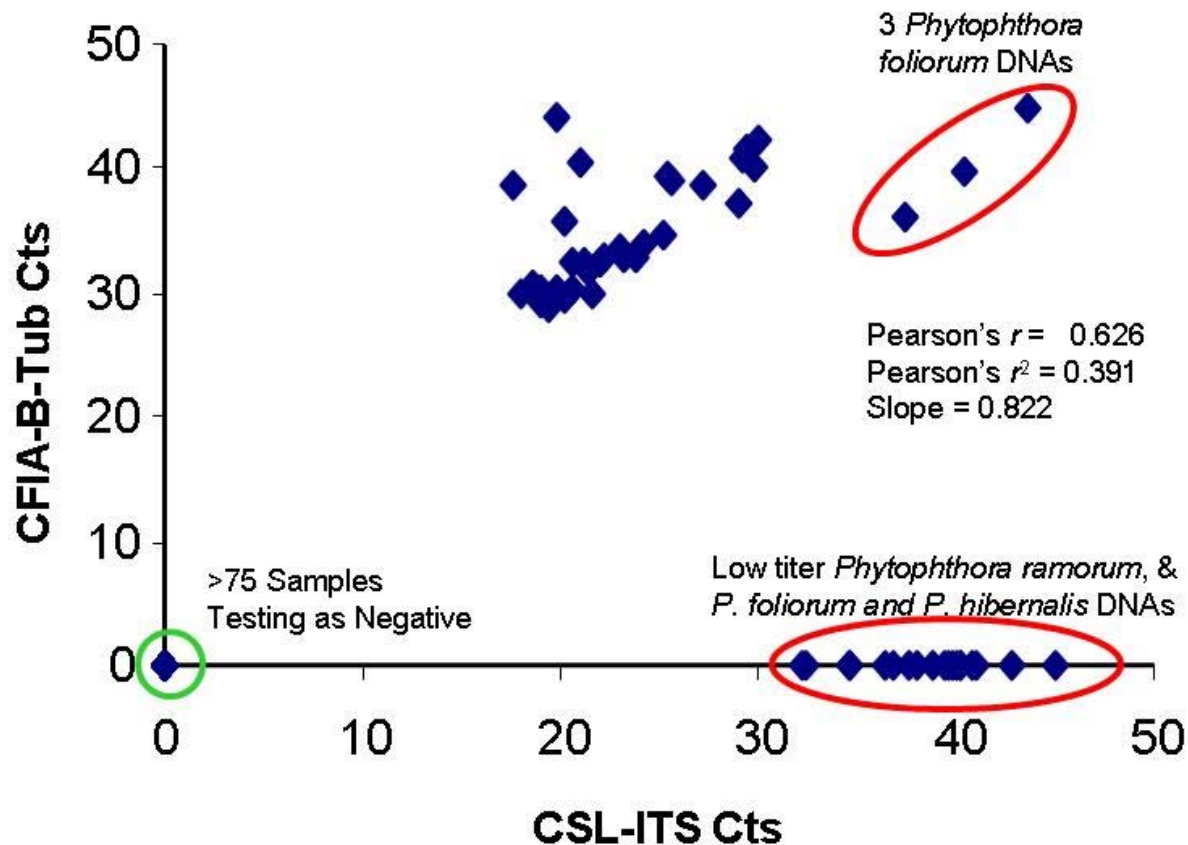
^c Tooley, Martin, Carras & Frederick 2006. *Phytopathology* 96:336-345

Samples Received at NPGBL for *Phytophthora ramorum* Diagnostics: 2004-2007

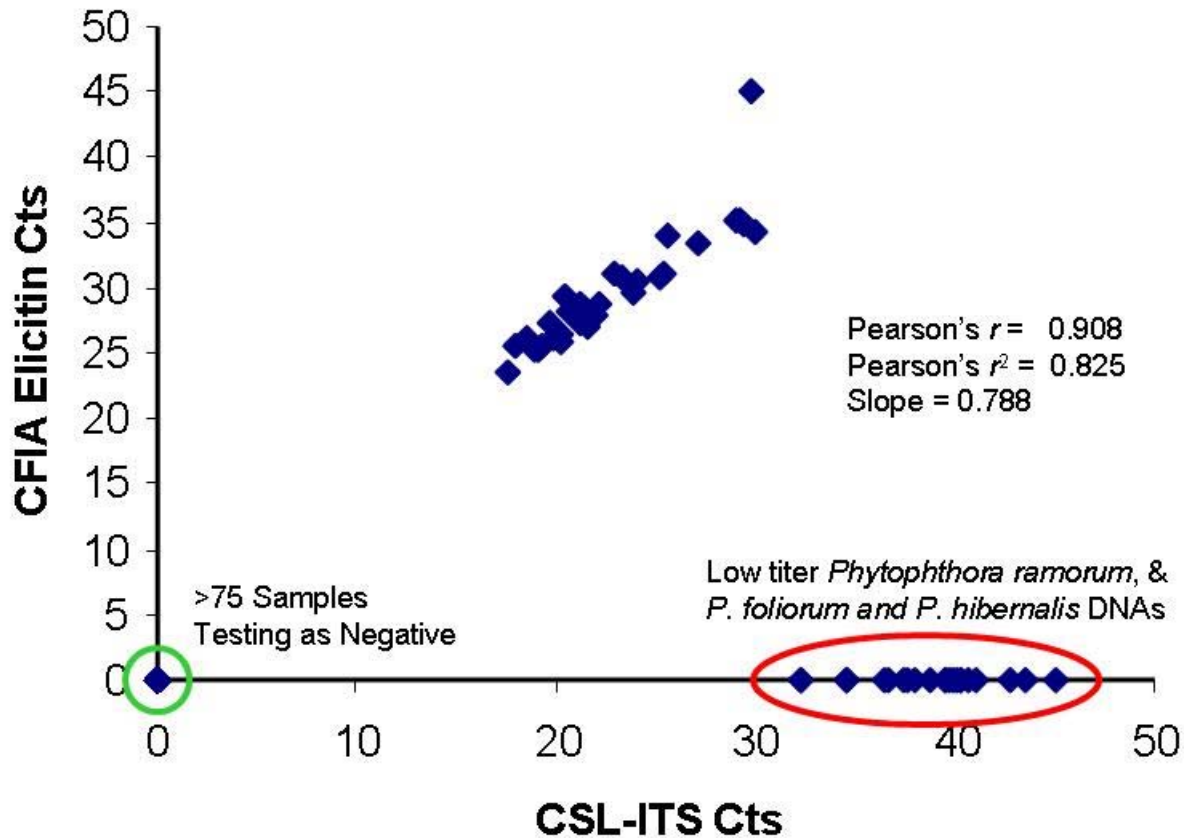
- 2004:
 - Over 3300 Samples received for testing
 - Approximately 320 PCR Positive (~9.6%)
- 2005:
 - Over 1800 Samples received for testing
 - 218 PCR Positive (~10.5%)
- 2006:
 - Over 1300 Samples received for testing
 - 166 PCR Positive (~14.3%)
- Early 2007*:
 - Over 300 Samples were received for testing
 - 54 PCR Positive (~17.7%)

* Responsibility for regulatory determinations of *P. ramorum* using PCR methods for PPQ was transitioned to PPQ-NIS-MDL in 2007

CSL-ITS Vs CFIA-B-Tub

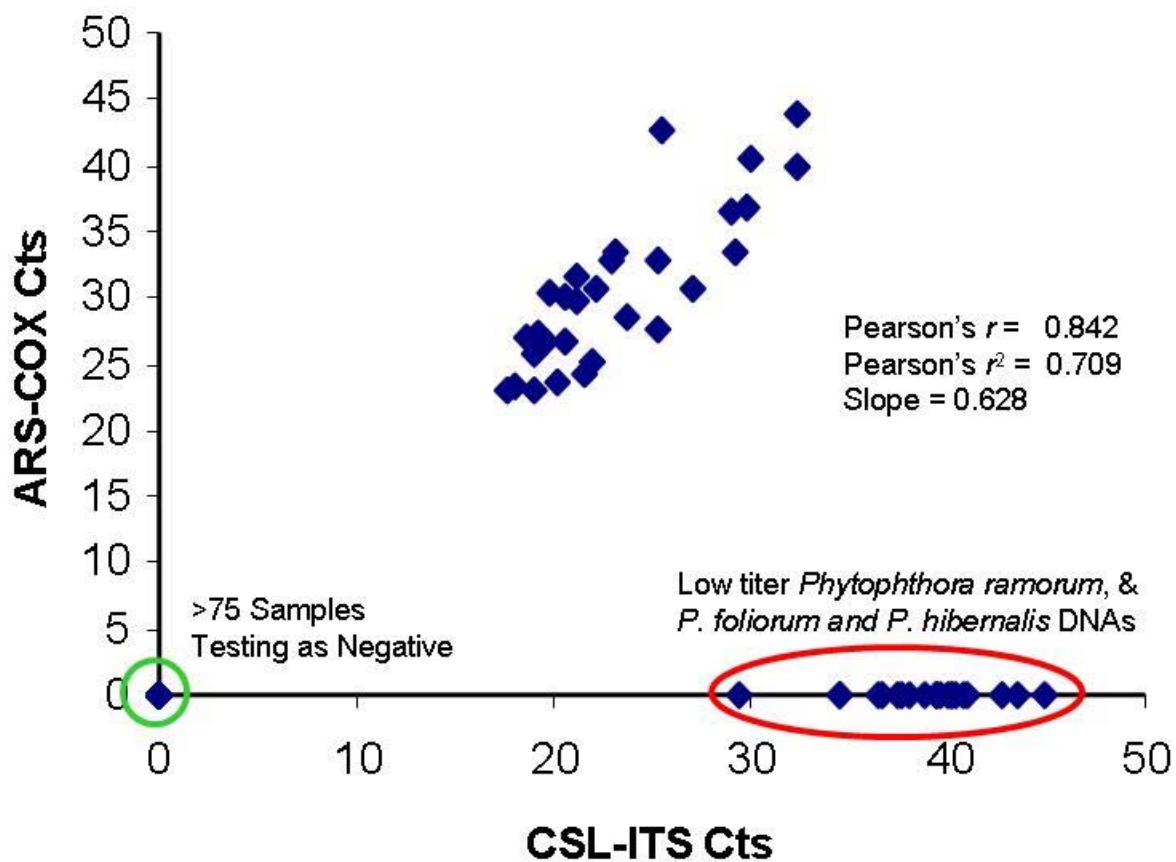


CSL-ITS Vs CFIA Elicitin



Plant Protection and Quarantine

CSL-ITS Vs ARS-COX



OPTION 1: No Change in Current System **(nested/multiplex conventional PCR + Real Time PCR)**

Advantages:

Highest sensitivity

Definitive identification by experienced labs

Current system in place

Some participating labs only use conventional PCR

Disadvantages:

Lower throughput/more time needed for assay

Requires more experience to ensure sample integrity

Requires more experience to interpret results

OPTION 2: Participating Labs Only Use Validated Real-time PCR

Advantages:

- Highest sensitivity of Real-time PCRs tested to date
- High throughput
- Part of current system in place

Disadvantages:

- Higher Potential for False Positives
- Most positive samples will need to be forwarded for confirmation
- Determination of low titer samples can't be made

OPTION 3: Participating Labs Use Validated Real-time PCR and CFIA Elicitin/5.8 S Assay

Advantages:

- Multiple genomic targets

- High throughput

- High specificity/low false positives

- Both plant DNA and "*Phytophthora*" DNA controls provide high quality assurance

- Good system for incorporation into an accreditation program

Disadvantages:

- Determination of some low titer samples can't be made and must be forwarded for confirmation

- Possible higher rate of false negatives

OPTION 4: Participating Labs Test Using Validated Real-time PCR and ARS Cox Assay

Advantages:

- Multiple genome targets
- Higher throughput
- High specificity/low false positives

Disadvantages:

- Determination of some low titer samples can't be made and must be forwarded for confirmation; higher potential for false negatives
- Multiplex control reactions redundant

CFIA Multiplex β -Tubulin & ITS Assays

Advantages:

- Multiple genome targets
- High throughput
- High sensitivity (ITS target)

Disadvantages:

- Cross-reactivity with related species (both targets)
- No internal control

For US Program - No Advantages to Current Diagnostics

CSL *Ypt1* Assay

Advantages:

- High throughput

- High specificity

Disadvantages:

- Not sensitive - higher rate of false negatives

- No internal control

Conclusions:

Current ITS-based validated Real-time PCR assay found to have higher sensitivity for *Phytophthora ramorum* than other tested assays

- can provide reliable determinations when used in concert with conventional PCR method

CFIA Elicitin and ARS Cox assays did not cross react with related or other tested *Phytophthoras*

Conclusions:

Because of the sensitivity, selectivity, and complementary control reactions, combined use of current ITS Real-time PCR and CFIA Elicitin/5.8S Real-time PCR for NPPLAP participating labs is recommended for incorporation into the current regulatory program

Recommendation to keep current conventional and Real-time PCR Work Instructions as an optional assay, if requested

Deployment of dual Real-time PCR diagnostic protocol to participating labs for testing/calibration in 2008

2009 PT panels will be designed for stand alone Real-time PCR