A PLANNED DEVIATION OF A CPHST-VALIDATED PROTOCOL ALLOWS FOR USE OF A HIGH-THROUGHPUT REAL-TIME MULTICYCLER FOR DETECTION OF *PHYTOPHTHORA RAMORUM*

***Elizabeth Bush*[1]**, *Geoffrey Dennis*[2], *Vessela Mavrodieva*[3] *and Mary Ann Hansen*[1]

[1]Virginia Tech, Blacksburg, VA, USA; [2]USDA-APHIS-PPQ S/T-NPPLAP, Raleigh, NC, USA; [3]USDA-APHIS-PPQ S/T-NPPLAP, Beltsville, MD, USA

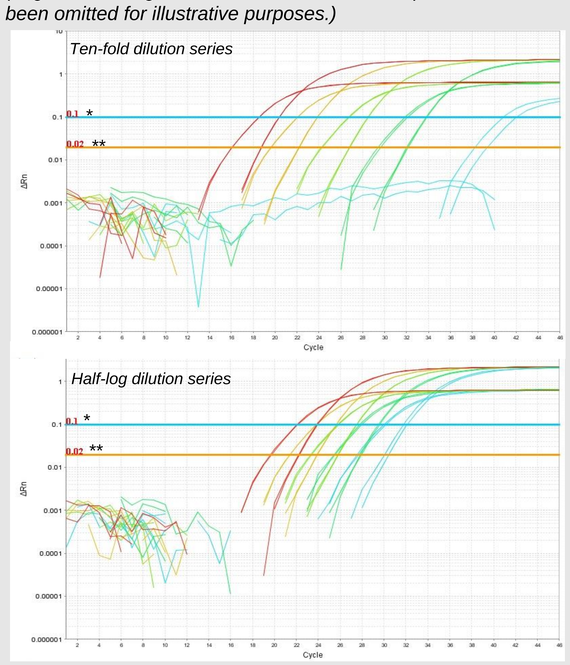
BACKGROUND

The Virginia Tech Plant Clinic (VTPC) desired to upgrade real-time PCR testing to a high-throughput platform, ABI StepOnePlus™, for *Phytophthora ramorum* detection. Technological advances have prompted NPDN and regulatory labs to seek replacement real-time platforms for the Cepheid SmartCycler®, currently used in PPQ-CPHST (Plant Protection and Quarantine-Center-Plant Health and Science Technology) *P. ramorum* protocols. National Plant Pathogen Laboratory Accreditation Program-accredited labs must use CPHST protocols for regulated pathogen screening. Successful performance of a planned deviation is necessary for any modification to a CPHST protocol in order to establish comparability to the original validated protocol. The VTPC developed a modified protocol for use on the StepOnePlus™ and a study was designed by APHIS-PPQ S/T-NPPLAP that would demonstrate comparability between the protocols.

PLANNED DEVIATION REAL-TIME PCR RUNS

Analysis of **PRECISION** relied on standard curve data generated from 10-fold and half-log dilution series of samples. Shown below are the standard curves generated from one *P. ramorum*-positive plant genomic DNA sample using the VTPC real-time protocol. Final precision results were reported as percent coefficient of variation.

(Log and half-log dilution series have been separated and the other standard curves generated during testing have been omitted for illustrative purposes.)

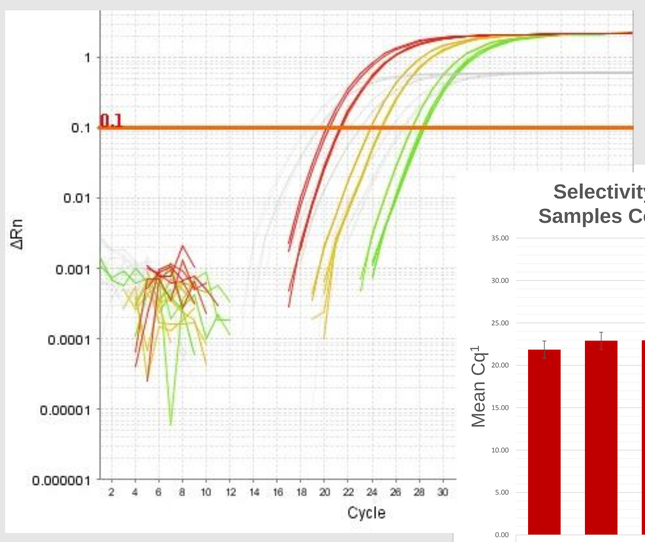


**Threshold for JOE reporter (plant COX gene target)*
***Threshold for FAM reporter (*P. ramorum *ITS target)*

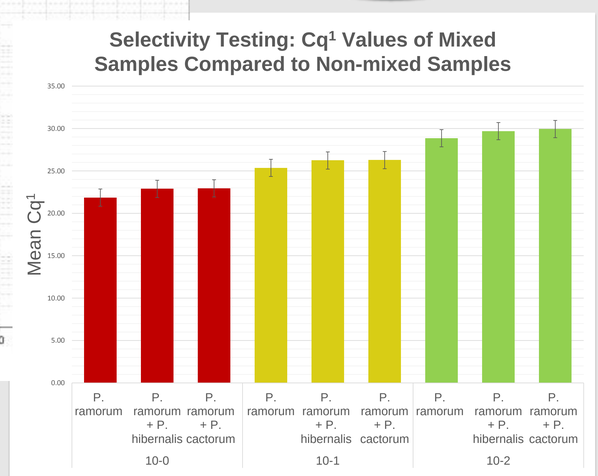
Actual Scope of Precision Testing for this Planned Deviation:

- ✓ Two different operators each performed a real-time run using two independently prepared dilution series.
- ✓ Two different *P. ramorum*-positive plant genomic DNA samples were used and real-time reactions (2 replicates) consisted of 10-fold and half-log dilution series.
- ✓ A total of 80 real-time PCR reactions were run for analysis of precision. Precision was also assessed during selectivity testing, for a total 176 reactions analyzed.

Analysis of **SELECTIVITY** relied on real-time standard curve data generated from dilutions (10^0, 10^{-1}, 10^{-2}) of *P. ramorum*/plant genomic DNA compared to mixed samples (1:1) of *P. ramorum*/plant genomic DNA and closely related *Phytophthora hibernalis* and distantly related *Phytophthora cactorum* genomic DNA.



NPPLAP-manufactured reference materials were used for all planned deviation tests



[1]*Cq=quantitative cycle. It is also commonly referred to as the "Ct" or "Ct-value"; however Cq is the preferred term and is currently the accepted industry standard, since the actual Ct-value output is typically normalized by an algorithm associated with the specific real-time machine (e.g. StepOnePlus®).*

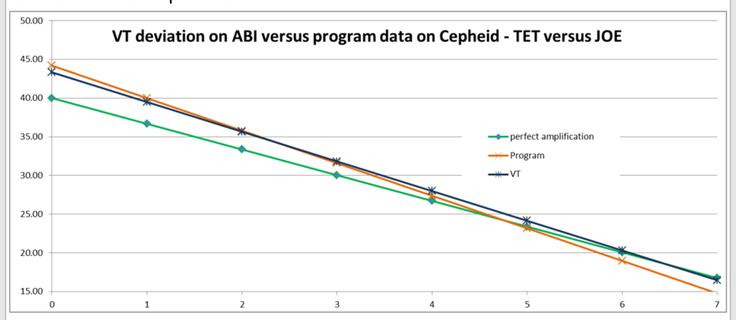
BEFORE BEGINNING PLANNED DEVIATION

- ✓ Develop protocol to favor chances of comparability while adopting technological advances in real-time chemistry
- ✓ Preliminary testing with minimal samples and parameters to establish: repeatability, range, and selectivity.
- ✓ Feasibility testing: Is the new protocol comparable to the original CPHST protocol?

THE PLANNED DEVIATION DEMONSTRATED COMPARABLE PRECISION, RANGE, DETECTION LIMIT AND SELECTIVITY FOR *P. RAMORUM* TO THE ORIGINAL PROTOCOL

Virginia Tech to Date	FAM	JOE	N	Total
Precision - Linearity	0.28%	0.58%	3	0.46%
Precision - Repeatability	0.65%	0.74%	32	0.70%
Precision - Intermediate	0.98%	1.09%	29	1.04%
Selectivity				99.43%
Linearity	0.9931	0.9908		
Accuracy	89.0%	82.3%		
Range Linearity Assessed	20-37	16-37		
Total N =				176
NPPLAP *P. ramorum* Proficiency Testing Program	**FAM**	**TxRd**		**Total**
Precision - Linearity	1.15%	1.34%		1.24%
Precision - Repeatability	2.02%	1.65%		1.85%
Precision - Intermediate	2.24%	2.00%		2.15%
Precision - Reproducibility	6.16%	6.11%		5.92%
Selectivity				99.38%
Linearity	0.9892	0.9887		
Accuracy	104.3%	75.2%		
Range Linearity Assessed	21-32	25-36		
Total N =				720

Normal regression (Cq-value to log dilution) analysis plot of plant internal control target (COX gene) including a plot of theoretical perfect amplification (slope = -3.32). The VTPC protocol was more accurate at high concentrations of plant material and directly comparable at mid and low concentrations to the original CPHST-validated protocol.



Definitions

- **Precision-Linearity**: Curve standard error.
- **Precision-Repeatability**: Variation among replicates in a single real-time PCR run.
- **Precision-Intermediate:** Observation of data points between different days, analysts (e.g. diagnosticians), instruments (e.g. SmartCycler® and StepOnePlus ®).
- **Precision-Reproducibility:** Observation of data points between different laboratories by the NPPLAP.
- **Selectivity:** Assessed by observing mixed reactions of *P. ramorum* and closely and distantly related *Phytophthora* spp.
- **Linearity:** Regression analysis of the standard curve reported using the coefficient of determination (r^2).
- **Accuracy:** Amplification efficiency of the standard curve.
- **Range Linearity Assessed:** The range of Ct-values assessed for determining the linearity of the assay.

After the Precision and Selectivity Results Were Approved:

- ✓ The NPPLAP proficiency panel for *P. ramorum* was performed successfully using VTPC protocol. Then the planned deviation was determined complete.
- ✓ The new protocol can be used by NPPLAP-laboratories for *P. ramorum* testing.

OUTCOMES OF THIS PLANNED DEVIATION

- Improvements to original protocol:
  - Uses commercially available real-time PCR kit for ease of use and signal normalization
  - Suitable for high-throughput *P. ramorum* detection
  - Increased assay performance: increased sensitivity over the original method.
- This successful process serves as a model for engaging NPPLAP member laboratories for other potential studies.

MORE WORK NECESSARY FOR FULL TRANSITION

- ➢ There are two real-time CPHST-validated *P. ramorum* diagnostic protocols (ITS and Elicitin targets) and performance of both are require to make a diagnostic determination for the NPPLAP *P. ramorum* Proficiency Testing Program.
- ➢ This planned deviation was for one of the CPHST-protocols (ITS target).
- ➢ We are beginning work on the second real-time protocol (Elicitin target) so that laboratories may fully transition to the ABI StepOnePlus®.