NPDN Interlaboratory Comparison Exercise (ILCE) For A New And Improved *Fusarium sambucinum* qPCR Assay.

Lina Rodriguez Salamanca[1], Elizabeth Bush[2], John Bonkowski [3], Sam Fieweger[4], Ana Cristina Fulladolsa Palma[5], Laura Miles[6],Steffie Rhodes [7], Alicyn Smart[8], Sumyya Waliullah[9] , Joe Viglienzoni[10], James Woodhall [11.]

(1)Iowa State University, Ames, IA, (2)Virginia Tech, Blacksburg, VA; (3) Purdue University, West Lafayette IN, (4)Wisconsin Department of Agriculture, Trade and Consumer Protection, Madison, WI, (5) Colorado State University, Fort Collins, CO (6) Michigan State University, East Lansing MI; (7) Michigan Department of Agriculture, East Lansing MI; (8)University of Maine, Orono, ME; (9) University of Georgia, Tifton, GA; (10) California Dept., of Food and Agriculture, Plant Health and Pest Prevention Services - Plant Pest Diagnostics Lab, Sacramento, CA; (11) The University of Idaho, Parma, ID.

Background

Diagnosticians in the National Plant Diagnostic Network (NPDN), including laboratories at land grant universities (LGU) and state departments of agriculture (SDA), face many challenges when choosing diagnostic assays to implement for pathogen detection in their laboratories. Although many testing methods are published and potentially available, these tests may lack complete validation data and, most importantly, interlaboratory comparison exercises (ILCE) that include various operators, equipment, and reagents. The NPDN Protocols and Validation Committee seeks to identify suitable diagnostic protocols for NPDN laboratories, establish best practices for method development and implementation, and undertake outreach efforts to promote diagnostic method validation and best delivery practices.

Fusarium sambucinum is a plant pathogen associated with dry rot in potatoes, canker and wilt in hops and also causes disease on strawberry, alfalfa, corn, and beans. The real-time PCR test used in this ILCE can be used to detect the *F. sambucinum* DNA extracted from pure cultures or plant tissue. The protocol was not designed for detecting *F. sambucinum* in DNA extracted from soil or other material. Performance of an ILCE as a component of the assay validation process, provides supporting data necessary for full validation that can be gathered by trained diagnosticians in their equipped laboratories.

Methods

In 2021, the first NPDN ILCE was conducted for a new qPCR TaqMan assay for *Fusarium sambucinum.* Twenty-four laboratories agreed participate. However, the exercise was completed by 19 laboratories, including 8 LGU NPDN diagnostic laboratories, 5 SDA laboratories, and 1 USDA-ARS and 5 LGU research labs (Table 1).

Two sample panels (differentiated by "yellow" or "purple") were prepared by the Plant Pathology and Diagnostics lab at the University of Idaho, Parma and shipped to participating labs. Each lab received two panels of eight samples and a known quantified positive control DNA. Panels consisted of target DNA at three levels, two blanks, and three with non-target Fusarium DNA (*Fusarium graminearum, Fusarium equiseti and Fusarium prolieferatum*). However, this information was not revealed to ILCE participating laboratories. Instead, each sample was labeled with a unique five-digit code.

Labs were asked to:

1. Order their own reagents, including primers and probes.
2. Quantify DNA concentration in each sample, prepare a standard curve from the positive DNA.
3. Determine the Limit of detection (LoD) and reaction efficiency of the standard curve.
4. Optional: include DNA from other *Fusarium* isolates or other soilborne pathogens from potato, corn or hops, in each run (see below), to test assay specificity.
5. Prepare three qPCR runs (plates): 1) Standard curve (LoD) alone, 2) purple panel samples and standard curve and 3) yellow panel samples and standard curve. Each standard curve point and unknown panel samples were run in triplicate (duplicate as a minimum) on a single plate.
6. Complete questionnaire (26 questions) on ILCE details and results. Data and any calculations (e.g LoD, reaction efficiency, etc.) were to be shown in the provided datasheet.

Results

Twenty labs returned results from March to September 2021. However, the data of one laboratory was not included since they did not provide any sample codes with their results and interpretation of results was not possible. One laboratory did not perform the exercise since they mistakenly ordered an MGB probe instead of the FAM-MGB stipulated with the protocol provided. Another two laboratories were unable to return the results due to other work commitments. The ILCE included a variety of qPCR brands and models (Table 1).

Table 1. List of participating laboratories types qPCR instruments models and brands that were utilized in the ILCE. DNA concentration reported by each laboratory, measured shortly after panel arrival and prior to plates preparation.

Lab No.	Laboratory type	qPCR machine brand and model	DNA positive control (ng/µl)*
1	University Research and Extension	Corbett Rotor-Gene	2.603
2	USDA Research	Agilent, AriaMx	14.1
3	NPDN, NCPDN	Biorad CFX96	10.75
4	NPDN, NEPDN	Applied Biosystems QuantStudio3	9.6
5	University Research and Extension	Biorad CFX96	11.5
6	State regulatory	Applied Biosystems QuantStudio5	10.7
7	NPDN, GPDN	Applied Biosystems QuantStudio3	ND
8	NPDN, NCPDN	Applied Biosystems QuantStudio3	10.3
9	University Research and Extension	Bio-Rad CFX	ND
10	State regulatory	Bio-Rad CFX	ND
11	State regulatory	Applied Biosystems QuantStudio3	ND
12	University Research and Diagnostics	Bio-Rad CFX 96	ND
13	University Research and extension	BioPhotometer D30, Eppendorf	15.05
14	NPDN, SCPDN	BioRad CFX Connect	12.8
15	NPDN, NEPDN	Applied Biosystems QuantStudio5	8.331
16	State regulatory	Biorad CFX96	12.7
17	State regulatory	Applied Biosystems QuantStudio 7 Flex	16.6
18	NPDN, NCPDN	Biorad CFX96	1.11**
19	NPDN, WPDN	Applied Biosystems QuantStudio3	11.7

*Concentration was measured by spectrophotometry (nanodrop) at participating laboratories. ** qubit used, instead of a nanodrop, ND: not measured.

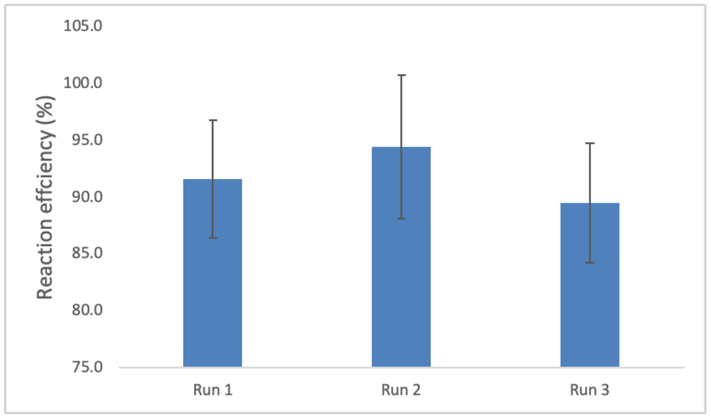

Fig.1. Reaction efficiency average of three independent qPCR runs. Values calculated based on the pooled data from all participating laboratories. Error bars represent standard error.

Table 2. Diagnostic parameters for the *F. sambucinum* qPCR assay

Diagnostic parameter	Panel 1 (purple)	Panel 2 (yellow)
Total datasets	19	19
Total data points	152	152
%True Positive	98	100
%True Negative	100	100
%False Negative	0	0
%False Positive	2	0

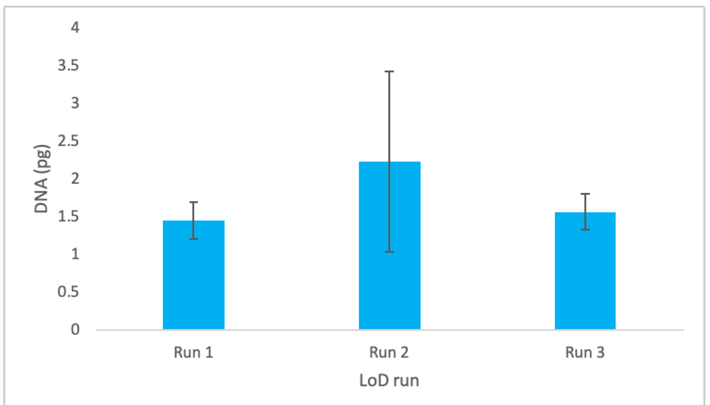

Fig.2. Limit of detection for the new *F. sambucinum* qPCR assay, normalized to the original positive standard concentration and pooled for all participating laboratories. Error bars represent standard error.

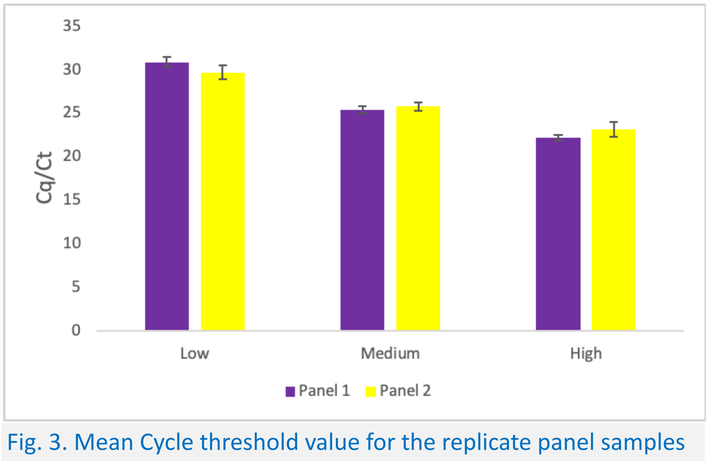

Fig. 3. Mean Cycle threshold value for the replicate panel samples spiked with low, medium and high levels of *Fusarium sambucinum*. Error bars represent standard error.

Acknowledgements

This work was funded by the NPDN Additional contributions funds (ACF) and the Northwest Potato Research Consortium. Special thanks to all ILCE participants who volunteered with and without ACF funds to be part of the exercise.

Reaction efficiency ranged from 75% to 110% with most values approximately 95% (Fig.1).

Specificity testing: Additional 25 *Fusarium spp.* isolates were included in panel runs by four labs. A cross-reaction was observed in one *Fusarium euwallaceae* isolate in one triplicate at Cq/Ct 39.4

One lab's results were not included, as they failed to provide original panel (tube) codes on their results. In addition, this lab was one to have false negatives. The other 18 labs had only three false positives from 180 negative samples over both panels (Table 2).

Since some laboratories did not quantify the positive DNA concentration (Table 1), the data presented for LoD quantification was normalized to the concentration measured at the Parma lab. Most laboratories' detection limit was at the standard dilution 10^{-5} or 10^{-6} dilution at approximately 2pg/ul of *F. sambucinum* DNA (Fig. 2). Six labs detected down to 0.234pg, six labs could inconsistently detect 0.234pg, and 5 labs consistently detected 2.34pg. For one lab they could only detect 23.4pg in one run but detected 2.34 pg in the other. Of the three samples positive for *F. sambucinum* in each panel, all labs were able to differentiate between the different levels of the target and there was consistency between the replicate panels for each lab and between labs (Fig. 3).

Conclusions

- Broadly consistent results between panels and between labs.
- The diagnostic parameters observed were very encouraging with only a 2% false-positive rate.
- Further ILCE should include more isolates per lab, for specificity testing.
- Guidance for ILCE statistical analysis, within the NPDN, will benefit current and future exercises.
- Many labs (24) were interested in participating, and many more showed interest during the 2022 GPDN webinar, which demonstrates the desire for fully validated diagnostic assays within the NPDN.
- As indicated by participants, the value of participating in ILCE is learning through experiential bench work and fellow diagnosticians' knowledge and experiences.
- Ordering the properly labeled probes is key, and a P&V tip article about qPCR probes' best practices is forthcoming in the NPDN communicator.
- ILCE can provide validation data and confidence in an assay, along with positive controls to laboratories, and help with assay implementation.
- Integrating an ILCE as a component for future assay development can promote best validation practices, facilitate rapid assay adoption, and foster collaboration between research and diagnostic labs. This approach may provide a useful model for future assay development, validation, and adoption within NPDN.