NPDN Protocols and Validation: Current Projects and Future directions.

Lina Rodriguez Salamanca[1]; James Woodhall[2a]; Margaret Moll[2b]; Sara R. May[3], Sam Fieweger[4]; MM (Mahfuz) Rahman[5]; Laura Miles[6]; Eva Grimme[7]; Joseph LaForest [8]; Dimitre Mollov [9], Steffie Rhodes[10]; Curtis Colbur [11]; Chandler Day[12]; Alicyn Smart[13].

(1) Iowa State University, Ames, IA; (2b) University of Idaho, Idaho Falls, ID;(2a) University of Idaho, Parma, ID; (3) The Pennsylvania State University, University Park, PA; (4) Wisconsin Department of Agriculture, Trade and Consumer Protection, Madison WI; (5) West Virginia University, Morgantown, WV; (6) Michigan State University, East Lansing MI; (7) Montana State University, Bozeman, MT; (8) University of Georgia, Tifton, GA; (9) USDA ARS, Corvallis, OR; (10) Michigan Department of Agriculture, East Lansing MI; (11) Clemson University, Pendleton, SC; (12) Kansas State University, Manhattan, KS; (13) University of Maine, Orono, ME.

Abstract

The NPDN Protocols and Validation committee focuses on identifying and addressing gaps in diagnostic assay validation data and improving diagnostic method development and implementation. We continue to engage with the broader scientific community to promote NPDN inclusion in assay development, validation, and diagnostic guide development. Our committee wishes to help inform diagnosticians when selecting and implementing published assays for immediate pathogen detection needs in their laboratories. To guide diagnosticians' choice of existing methods, we propose: (1) validation levels to rank assays and (2) validation-verification internal forms to document the implementation process and categorize a particular assay within a validation level. Both proposals are presented in this poster and open to the NPDN members for commentary and suggestions. Our committee is extending invitations to develop key diagnostic guides to researchers, and we have created a set of recommendations for diagnostic guide manuscript development. These recommendations include a thorough review of diagnostic workflow and requests that authors rank proper screening and confirmatory diagnostic methods and assign validation levels to methods included in the guide. Also, we advise the inclusion of diagnosticians in developing diagnostic guides to provide insight from the perspective of working in the diagnostic setting, often with suboptimal samples. Lastly, our committee is committed to creating best practice documents helpful to NPDN members within the framework of providing quality services and identifying future needs for best practice documents related to quality management systems.

Validation level proposal

The validation levels and terms proposed below will be used to rank different diagnostic assays, these terms may change over time if: (1) a new test performance comparisons paper emerges, (2) the population of the pathogen changes, and the test performance is compromised, (3) a new and/or improved test (s) is/are developed and properly validated.

Gold (enhanced) validation: Assay published with in-depth specificity (at least 35 isolates) and sensitivity testing (technical and all relevant biological replicates); comparison of the standard test with at least 100 samples including naturally infected matrixes; inter-lab comparison or ring test; all pertinent matrixes tested; and reproducibility and repeatability data (three times) is provided.

Silver (standard) validation: Assay published with specificity (at least key 20 isolates) and sensitivity testing (technical and the key biological replicates); comparison of the standard test with a limited number of samples; key matrixes tested; proof it works with naturally infected samples; and reproducibility and repeatability data (three times) is provided.

Minimum validation. Assay may or may not be published, basic specificity test with less than 20 isolates but in silico testing of additional isolates completed; technical sensitivity; proof it works on real-life samples; and basic reproducibility and repeatability (twice) provided. Minimum validation is considered the lowest tier of validation and will eventually go away but is used when assay validation data is insufficient.

In-house lab verification: Used for assays that lack basic validation data in publication. The verification form proposed is used to guide in-house verification and includes in silico testing of spiked host samples, plant internal controls, and minimum standards for validation parameters of sensitivity, repeatability, reproducibility, and robustness.

Diagnostic guide recommendations

Greenhouse and Nursery Plants Sample Processing for Diagnosis- Best Practices

NPDN members your feedback is appreciated

Leave us your comments, questions at https://padlet.com/lina253/PandV

NPDN National Plant Diagnostic Network

Verification form draft

Fieweger, Miles, Rodriguez P&V Best practices

Verification data for diagnostic methods

Verification: Confirmation by examining objective evidence that specified requirements have been fulfilled [1,2]. In the context of this form, a new assay is being implemented in the lab and compared with a prior one (if applicable).

1. **Laboratory information**
   a. Name and contact:
   b. Is the lab accredited for this test? Y/N
   c. If yes, list lab accreditations (core, STAR D?, CORE, ISO, other)

2. **Identify the scope of the test**
   2.1. Target Organism
   Please give species name and/or strain details of the target

   2.2. Detection/ identification or both (type):

   2.3. Matrix(ces): Select the type of matrice(s) tested during the validation process
   Leaves, Shoots, Fruits, Seeds, Roots, Herbaceous cuttings, Woody cuttings, Wood, Bark, Tubers, Bulbs, Pure culture, Soil, Water, Specimen, Other:

   2.4. Plant species tested (use common and *scientific names*):

   2.5. SOP/WI number(s)

   2.6. Origin of assay
   Give project details and/or publication details

   2.7. Method (select one, full list of definitions, see https://npdn.ceris.purdue.edu/lab_method.php)
   Bioassay, Biochemical, Culturing, Conventional PCR (cPCR), Electron Microscopy, ELISA, Image, Inclusion Body, Incubation, Isothermal amplification, Lateral Flow Device (LFD), Molecular (Other), Microscopic, Nematode Extraction, Next-generation sequencing, Polymerase Chain Reaction, Real-time PCR, Sequencing, Soil Analysis, Serological (Other), Tissue Analysis, Visual Observation

   2.8. Assay details
   2.8.1. Nucleic acid extraction (if applicable), answer the following questions; Modified and compared with a reference test?
   Kit used? (Manufacturer names, name of the kit, specify any modifications from manufacturer instructions)
   Nucleic acid extraction:
   Sensitivity: Extract three subsamples at the established smallest amount of tissue (usually 50-100 mg). Extracts should contain enough amount of high-quality nucleic acid to obtain reliable results in downstream diagnostic methods.
   Specificity: N/A, extracts will contain total DNA, RNA or a combination of both.
   Selectivity: Determine if variations in the matrix (type of tissues, quality of tissue, etc) affect the performance of the test.
   Repeatability: Extract three replicates per sample. If consistent results (in terms of nucleic acid concentration, purity and integrity) are not obtained, additional replicates should be performed.

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Reproducibility: Extract three replicates per sample. Each replicate should be extracted at different times and when possible, with different operators
Robustness: Replicate extractions should be performed in altered test conditions to determine if the altered conditions affect the test.

2.8.2. PCR based methods-Please detail: Target, primer/probe names, probe modifications, sequences and target region, type (simplex, duplex, multiplex, nested etc)

Primer/probe name	Sequence (5' - 3')	Target region	Source

2.8.3. Reaction conditions
Include reagents, concentration, quantities, instruments (homogenizer, liquid handler/ NA extraction, fluorometer, plate reader, thermocycler etc) used and cycling conditions

Performance criteria

3. **Specificity (inclusivity and exclusivity):** Select a few of the most relevant targets (e.g. different strains, populations) for inclusivity and non-targets for exclusivity available to the laboratory. Select a few of the most relevant targets (e.g. different strains, local isolates) and non-targets (10) common to host/matrix. Tests should be performed at the middle of the detectable range reported for the assay.

Describe the outline of the experiment undertaken to determine the specificity of the assay. List of controls (positive, host negative) and any other material available, artificial positive controls (APC), plasmids or oligos. If PCR inhibition due to host matrix is expected, spike host negative material with APCs to evaluation inhibition.

cPCR: Purchase/ obtain negative/healthy control (be aware of matrix).If the pathogen population is not being detected (confirmed in a sample by secondary method), obtain geographically different samples to check.
Real time PCR: NA or maybe running a limited specificity test if possible
ELISA: Purchase/ obtain negative/healthy control (be aware of host and tissue type). if the pathogen population is not being detected, obtain geographically different samples to check

4. **Sensitivity (technical and biological):** Analyse at least eight samples at the established limit of detection. This can be combined with repeatability/reproducibility.

Describe the outline of an experiment undertaken to determine the sensitivity of the assay. PCR-based methods: Minimum requirements are to analyse at least 6–10 DNA samples (preferably in triplicate) and obtain the technical limit of detection (L.O.D) in 'pg DNA per µl'. Please state the nucleic acid extraction method used to produce the DNA extract.
Calculate the reaction efficiency of the assay with pure DNA.
If possible, also determine sensitivity prepared by spiking the target into a specific substrate to produce the limit of detection for the target within the substrate. Give sensitivity in appropriate units e.g. cyst per kg of soil.

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NPDN P&V Committee Suggestions for Diagnostic Guides

Below are some ideas, most useful information for diagnosticians, currently not required or mentioned in the "Plant Health Progress Instructions for Authors":

Sampling
- Appropriate matrix (e.g., plant parts to sample, soil sampling specifics, etc.) and the amount needed for screening or confirmatory tests (culture, serology, molecular, etc.), ideal phenology/age, shipping time, refrigeration/ice, etc., as applicable.
- Encourage authors to partner with diagnosticians in their state or region for working with suboptimal samples and provide input on the information below as needed
- Remind field practitioners of the importance of taking site information and digital photos while sampling. Encourage authors to include examples in the symptoms section.

Symptoms section:
- Lookalike problems/ confused with (list, collage of images, and or link to bugwood images or collections or relevant resources, see to learn more about bugwood images see this article)
- Key times/phenology/model to scout for symptoms (if applicable)
- Symptom progression images
- Varietal differences
- Brief environmental and conditions conducive to disease (weather, growing practices, etc.)
- Pattern in plant
  - Pattern in field/greenhouse/ landscape

Pathogen isolation section:
- ***Tissue surface disinfestation*** recommendations: wash (soap and water?), rinse, alcohol (dip/flame), bleach and concentration, rinse (for how long?). Is isolation impacted by plant age/tissue type?

- ***Culture incubation*** parameters: temp, light, timing

Pathogen identification section:
- ***Incubation*** (moist chamber): Timing (hours, days), surface disinfestation recommended or not.
- Indirect vs direct humidity (e.g. soft tissues should not be incubated with direct humidity)

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Greenhouse and Nursery Plants Sample Processing for Diagnosis- Best Practices

By Jan Byrne[1], Tom Creswell[2], Sara May[3], Mike Munster[4], Melodie Putnam[5], and Lina Rodriguez Salamanca[6].

1)Michigan State University, 2) Purdue University, 3)The Pennsylvania State University, 4) North Carolina State University, 5) Oregon State University, and 6) Iowa State University.

Sample and information

- Request whole plants and multiple plants expressing a range of disease severity. Inquire for clients to collect sample before spraying pesticides.
- Request full flats (or a section thereof - minimum of 6 cells) if seedlings
- Request genus, species and cultivar names.
- Request photos, patterns, percentage of plants affected, and as much information on greenhouse conditions as possible. Relevant information includes:
  - Seed vs. vegetative propagation,
  - Recent (last 30 days) applications of fertilizers, pesticides, and PGRs
  - GH temperature records (if available), cooling- airflow system.
  - Heater type
  - Irrigation water source, method of application, pH and alkalinity; type of recirculation system,
  - Shade cloth? are nursery plants overwintered undercover or our in the open (depends on plant and location)
- Paraphrase some of the questions from this list http://egrouni.com/pdf/2018_710.pdf as applicable.
- Provide instructions for how to properly package and ship samples so they won't be destroyed in the mail. Ship overnight (preferred) or 2-day delivery (max.).

Sample inspection

- Carefully read and consider the information provided by the client.
- Consider making checklists of common problems in your area on commonly received kinds of plants.
- Always look before rinsing anything off. You may wash away a clue.
- Plants with the most advanced symptoms are more likely to have secondary organisms present so begin the examination with those showing intermediate symptoms

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Nematode extraction: The method should be able to extract a sufficient quantity of the target organism. If possible spiked samples should be used to determine the percentage of nematodes recovered.
cPCR: First, run a positive and negative control (full concentration) in triplicate to see if assay works. If it does (i.e. band presence/ brightness and expected size), Analyze (at least three experiments?) serial dilutions of the positive and negative control to determine detection limit.
Real time PCR: First, run a positive and negative control (full concentration) in triplicate to see if assay works. If it does (i.e. getting similar Ct's [not greater than 3.5 Ct's) to those reported), analyze (at least three experiments?) serial dilutions of the positive and negative control to determine the detection limit. OR just one experiment with triplicate dilutions
ELISA: Include serial dilution of positive control, in duplicate per kit or positive control lot, when starting a new one.

5. **Selectivity:** The extent to which variations in the matrix affect the test performance (matrix effect)
   Determine the relative insensitivity of the test to variations of the sample material, e.g. different cultivars of the host plant, different parts of the host plant (roots, leaves, stem etc).
   List matrices tested & nucleic acid extraction method used.

Nematode extraction: If using different matrix from validated test develop and plant a deviation study.
cPCR and Real time PCR: If testing different matrix (tissue) develop plan deviation study (using different tissue not intended in test). Include internal amplification control
ELISA: If testing different matrix (tissue) develop plan deviation study if using different tissue not intended in the kit instructions. (non ideal samples).

6. **Repeatability:** Level of agreement between replicates of a sample tested under the same conditions

Perform at least 2 experiments with three levels of target organism (low/medium/high) and at least eight replicates with the same operator and equipment at the same day. The 'low' level should be at or approaching the limit of detection.

PCR based: Repeat analytical sensitivity test 3 times (3 separate tests) only with lower dilution.
ELISA: Prior extracts serial dilutions, in three different tests, use lower dilutions only (based on sensitivity test)

7. **Reproducibility**

Perform at least 2 experiments with 3 levels of target organism (low/medium/high) but at different moments, when possible with different operators, and when relevant with different equipment. This can be combined with repeatability testing. Again, at least eight replicates should be tested.
It is recommended that at least one operator works within a diagnostic team.

Nematode extraction: Extraction should be performed by another diagnostician/lab technician-assistant.
PCR based: Run by other diagnostician/ lab assistant.
ELISA: When assesssing a new assay in the lab all potential operators should perform the assay with results to be compared between all operators. When new operators enter the lab they should perform the assay and have results compared to existing results. Same for plate readers (equipment). Keep prior extracts in -80oC.

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8. **Comparison with a 'standard test'**
   Please compare the new test in conjunction with any existing test to benchmark performance. Determine % agreement of new test with standard test.

		Standard test		
New test		+	-	Total
	+	PA	PD	
	-	ND	NA	
	Total			

PA, positive agreement; PD, positive deviation; ND, negative deviation; NA, negative agreement.
Positive (+) and negative (-) results for (90 - 100 for validation of new test) samples tested using both tests (subsamples are acceptable), illustrating diagnostic sensitivity [PA/(PA+ND)], diagnostic specificity (NA/(NA+PD)), and relative accuracy (PA+NA)/(PA+PD+ND+NA).

9. **Interlab comparison exercise/ test performance study (optional)**
Have the results been produced as part of a NPDN/ Interlab comparison exercise (ILCE), Ring test (either type of Test Performance Study (TPS))?
Provide details, It is available, link to published article/report.

10. **Long term monitoring**
Describe the frequency and method(s) of checking required.

11. **Statement on test verification**

Performance criteria	Values from the standard test (if applicable)	Values from the new test	Where to find documentation (records)
Analytical sensitivity Analytical specificity Selectivity Repeatability Reproducibility			

On the basis of the above statement, the verification of the test is judged suitable for the scope of the test.

Name in capital letters:	Laboratory position:
Signature:	Date:
Name in capital letters:	Laboratory position:
Signature:	Date:

[1] Diagnostic Assay Validation Terminology.
https://www.apsnet.org/edcenter/disimpactmngmnt/Pages/AssayValidationGlossary.aspx

[2]PM 7/98 (5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity https://doi.org/10.1111/epp.12780

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