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White-Nose Syndrome Diagnostic Laboratory Network Handbook

Section E, Mammalian Diseases
Book 15, Field Manual of Wildlife Diseases

Techniques and Methods 15–E1

U.S. Department of the Interior
U.S. Geological Survey

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By Katrina Alger and the White Nose Syndrome National Response Team Diagnostic Working Group

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U.S. Geological Survey

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The White-Nose Syndrome Diagnostic Working Group Members

Members are from the time work was completed in alphabetical order.

Anne Ballmann, Group Chair, U.S. Geological Survey National Wildlife Health Center

Robert Bildfell, Oregon State University Veterinary Diagnostic Laboratory (retired)

David Blehert, U.S. Geological Survey National Wildlife Health Center

Steven Bolin, University of Michigan

Elizabeth Buckles, Cornell University Animal Health Diagnostic Center

Christopher Cornelison, Kennesaw State University

Colleen Duncan, Colorado State University Veterinary Diagnostic Laboratories

Heather Fenton, Canadian Wildlife Health Cooperative

Jeffrey Foster, Northern Arizona University Pathogen and Microbiome Institute

Winifred Frick, Bat Conservation International

Kyle George, U.S. Geological Survey National Wildlife Health Center

Colin Gillin, Oregon Department of Fish and Wildlife

Andree Hunkapiller, Oregon State University Veterinary Diagnostic Laboratory

Kevin Keel, University of California, Davis

Patrice Klein, U.S. Forest Service

Daniel Lindner, U.S. Forest Service

Jeffrey Lorch, U.S. Geological Survey National Wildlife Health Center

Scott McBurney, Canadian Wildlife Health Cooperative

Donna Mulrooney, Oregon State University Veterinary Diagnostic Laboratory

Nicole Nemeth, Southeastern Cooperative Wildlife Disease Study

Daniel O’Brien, Michigan Department of Natural Resources

Kristy Pabilonia, Colorado State University Veterinary Diagnostic Laboratory

Katie Parise, Northern Arizona University Pathogen and Microbiome Institute

Justin Sanders, Oregon State University Veterinary Diagnostic Laboratory

Terry Spraker, Colorado State University Veterinary Diagnostic Laboratories

Michelle Verant, U.S. National Park Service

Julie Weckworth, U.S. Forest Service

Daniel Wilson, U.S. Forest Service

Conversion Factors

International System of Units to U.S. customary units

Multiply	By	To obtain
	Length	
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
	Volume	
liter (L)	33.81402	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
microliter (µL)	0.0002029	teaspoon (tsp.)
milliliter (mL)	0.2029	teaspoon (tsp.)
	Mass	
kilogram (kg)	2.205	pound avoirdupois (lb)
gram (g)	0.03527	ounce, avoirdupois (oz)
milligram (mg)	3.527×10^{-5}	ounce, avoirdupois (oz)
nanogram (ng)	3.527×10^{-9}	ounce, avoirdupois (oz)
femtogram (fg)	3.527×10^{-15}	ounce, avoirdupois (oz)
attogram (ag)	3.527×10^{-18}	ounce, avoirdupois (oz)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

°F = (1.8 × °C) + 32.

Abbreviations

APHIS	Animal and Plant Health Inspection Service
BSC	biological safety cabinet
BSL	biosafety level
CDC	Center for Disease Control and Prevention
Ct	cycle threshold
CV	coefficient of variation
DNA	deoxyribonucleic acid
DSe	diagnostic sensitivity
DSp	diagnostic specificity
DxWG	Diagnostic Working Group
IPC	internal positive control
LOD	limit of detection
LOQ	limit of quantification
NAHLN	National Animal Health Laboratory Network
Pd	*Pseudogymnoascus destructans*
PHAC	Public Health Agency of Canada
PPE	personal protective equipment
QMS	quality management system
qPCR	quantitative polymerase chain reaction
USDA	U.S. Department of Agriculture
USFWS	U.S. Fish and Wildlife Service
USGS	U.S. Geological Survey
UV	ultraviolet
WHO	World Health Organization
WNS	white-nose syndrome
WOAH	World Organisation for Animal Health

White-Nose Syndrome Diagnostic Laboratory Network Handbook

By Katrina Alger and the White Nose Syndrome National Response Team Diagnostic Working Group

Abstract

When responding to a wildlife disease outbreak, managers depend on consistent and clear data to make decisions. However, diagnostic methods for detecting pathogens of wildlife often lack the level of procedural and interpretational standardization that occurs in the investigation of human and domestic animal diseases. This lack of standardization can hamper diagnostic reliability in two ways. First is the inappropriate application of tests to new species or in situations that are outside of the original (in other words, validated) purpose. Second is the use of laboratory-specific modifications or analytical parameters without thorough investigation of how those changes affect result comparisons across institutions or the ability to make broader conclusions about pathogen or disease.

White-nose syndrome (WNS) is a disease caused by the fungal pathogen *Pseudogymnoascus destructans* (*Pd*), which has spread rapidly and is causing population-level declines in some species of North American bats. During the last decade, quantitative polymerase chain reaction (qPCR) has become the most common method of testing for *Pd* because of qPCR's speed, accuracy, and simplicity across a wide range of invasive and noninvasive sample types. Its widespread use by many State, Federal, Provincial, and academic institutions has inevitably led to variations in methodology and interpretation among laboratories. The progressive geographic spread of fungus and disease has also led to sampling contexts and strategies that differ from those for which the qPCR assay was originally developed and validated. These factors have resulted in inconsistencies among results tested in different laboratories and, subsequently, confusion for managers and decision makers.

To address these challenges, the WNS National Response Team Diagnostic Working Group launched a project congruent with increased calls for the harmonization of wildlife disease diagnostic results, and reporting standards across disparate methodologies and laboratories. Beginning in 2019, interlaboratory testing was done to better understand how variations to *Pd* qPCR methodology affect diagnostic consistency and to reassess the assay's fit for purpose in new testing contexts. This information led to expanded conversations within the Diagnostic Working Group related to best practices in *Pd* qPCR diagnostic testing, the development of common interpretation language for classifying test results, and the incorporation of that language into an updated WNS case definition. This handbook is the resulting product and is intended to help further harmonize *Pd* qPCR diagnostic testing by establishing recommendations related to voluntary participation in a WNS Diagnostic Laboratory Network, documenting the currently (2022) practiced *Pd* qPCR methodologies, discussing general best practices for molecular diagnostics and laboratory networks, and elaborating on the epidemiologic and diagnostic basis of the agreed-upon classification language for *Pd* qPCR results. Through this voluntary, consensus-based approach to diagnostic harmonization, this work aims to improve the confidence of management agencies in reported *Pd* qPCR results and can serve as an example of national diagnostic coordination for other unregulated wildlife diseases.

Introduction

Diagnostic testing plays a fundamental role in disease surveillance and management. National disease diagnostic laboratory networks, and the subsequent coordination of laboratory diagnostic methods are well established for human (Centers for Disease Control and Prevention [CDC]; World Health Organization [WHO]) and domestic/agricultural animal health (U.S. Department of Agriculture [USDA]; World Organisation for Animal Health [WOAH], formerly known as Office International des Epizooties [OIE]). In these fields, multiple laboratories form a network under the oversight of a governing agency, typically at the national or international level. These laboratory networks harmonize or standardize requirements such as diagnostic protocols, quality control, proficiency testing, reporting standards, and communication protocols to provide confidence in diagnostic test results for infectious diseases of humans or domestic animals that, if detected, may have substantial economic or social consequences.

When responding to wildlife disease outbreaks, natural resource managers also depend on clear and consistent diagnostic information to guide their decision-making process (fig. 1). Lack of regulatory authority for nonreportable wildlife diseases means that standardized methods for detecting wildlife pathogens, including consistent criteria for interpreting

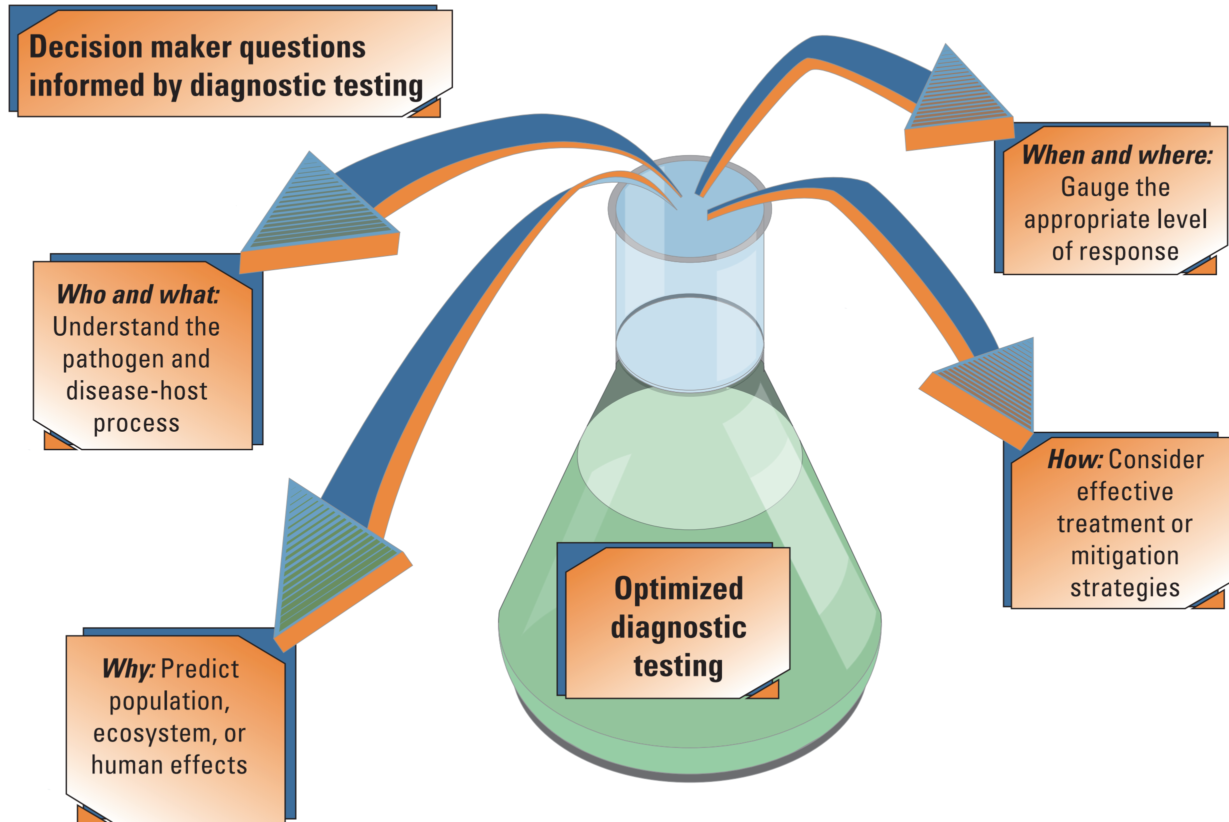


Figure 1. Decision-maker questions that can be informed by diagnostic testing.

and communicating results, are not always available (box 1; Sleeman and others, 2019). Testing is often done by laboratories across States, Provinces, Federal agencies, and academic institutions, all of which may perform tests for a pathogen using different equipment, reagents, methodologies, or interpretation standards. These variations can subsequently cause confusion when results communicated to managers by different laboratories are inconsistent.

White-Nose Syndrome Response Team Diagnostic and Surveillance Working Group

White-nose syndrome (WNS) is a disease caused by the fungal pathogen *Pseudogymnoascus destructans* (*Pd*) that has spread rapidly and is causing population-level declines in some species of North American bats. The fungus and the disease have moved rapidly across the country since they were first reported in New York in 2006 (Blehert and others, 2011). The White-Nose Syndrome National Response Team is a coordinated, interdisciplinary, and multiagency effort led by the U.S. Fish and Wildlife Service (USFWS) to "conserve and strengthen healthy bat populations in the face of the disease" (WNS Response Team, 2022). The response consists of an Executive Committee, a Steering Committee, a Stakeholder Committee, a Technical Review Committee, and five working groups: Surveillance and Diagnostics, Data Management, Conservation and Recovery, Communications and Outreach, and Disease Management.

Before the merging of the Diagnostic and Surveillance Working Groups, the Diagnostic Working Group (DxWG) provided coordination between laboratories processing WNS samples, assessed sample-processing capacity within the WNS Laboratory Network, and promoted timely result reporting to managers and researchers (WNS Response Team, 2014). This was achieved through interlaboratory collaboration and communication, sharing of protocols for collecting and submitting samples, supporting laboratories interested in developing testing capacity, and publishing methods in the peer-reviewed literature. Various members of the DxWG participated in the development of diagnostic methods for WNS (the disease) and for *Pd* (the causative pathogen) that are still widely used today including ultraviolet (UV) fluorescence, histopathology, and fungal culture (Meteyer and others, 2009; Turner and others, 2014). Molecular methods such as conventional and real-time quantitative polymerase chain reaction (qPCR) and genetic sequencing added the ability to detect and identify genetic

Box 1. Wildlife Disease Reporting

Animal health professionals are required to report the detection of certain pathogens and diseases because of their potential ability to cause illness or disease in humans (zoonotic agents) or their threat to agricultural production. The World Organisation for Animal Health (WOAH) and the U.S. Department of Agriculture Animal and Plant Health Inspection Services maintain lists of pathogens and diseases for which they require immediate notification when diagnosed in either domestic or wild animals. To facilitate accurate and timely reporting, WOAH maintains detailed case definitions for each notifiable disease that include information related to diagnostics and result interpretation and are reviewed on a regular basis as part of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/). In 2020, the Animal and Plant Health Inspection Services proposed the creation of a regulatory National List of Reportable Animal Diseases (https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/monitoring-and-surveillance/nlrad/ct_national_list_reportable_animal_diseases) to legally mandate compliance with existing reporting requirements and better align with WOAH standards for monitoring and managing certain pathogens.

For wildlife diseases with no known direct effect on domestic animal or human health, reporting has typically not been required either nationally or internationally. Diagnostic and interpretation guidance for these nonreportable diseases is typically developed for individual institutions and can lack consistency, which hinders large-scale response or mitigation. This anthropocentric definition of what makes a disease reportable is changing, however, as recognition grows regarding the threat of disease to wildlife conservation efforts. For example, in 2010 the WOAH added two amphibian pathogens—ranavirus and *Batrachochytrium dendrobatidis*—to their list of notifiable agents followed by *Batrachochytrium salamandrivorans* in 2019. Despite the fact these pathogens pose no known human or agricultural risks, their effect on global amphibian populations is undisputed, and the increased risk of transboundary spread through the pet and food trades contributed to a need for the more formal regulatory structure achievable through WOAH listing (Schloegel and others, 2010).

material from both viable and nonviable fungus and quickly became the preferred diagnostic method for large-scale *Pd* surveillance throughout the United States and Canada (Lorch and others, 2010; Minnis and Lindner, 2013; Muller and others, 2013; Inter-agency White-Nose Syndrome Committee, 2015).

As the pathogen and disease have moved westward in North America (fig. 2), surveillance for *Pd* using PCR and qPCR has also expanded beyond approaches used in the initial phase of the epizootic. This has included sampling new species in different habitats, increased reliance on new sample matrices such as cave sediment and bat guano, and collecting samples outside of hibernation season when fungal loads are typically highest and signs of WNS tend to manifest. Consequently, samples from a particular site may test PCR-positive for the *Pd* fungus without observation of WNS clinical signs within the sampled population or, in some cases, in absence of observable host species. In parallel with these changes in sampling strategies, laboratories have increasingly reported test results that indicate the presence of very small amounts of target genetic material (in this case, deoxyribonucleic acid [DNA]) in a sample, raising questions about how these results should be interpreted and what their biological or ecological significance may be. Although many laboratories testing samples for *Pd* are using the same qPCR assay (Muller and others, 2013), changes in sampling from sites with high bat mortality and high pathogen loads to sites with little or no mortality and presumed low amounts of fungus present has created challenges for managers when it comes to result interpretation.

The WNS DxWG has taken several steps to address these challenges, starting with the establishment of a more formalized WNS Diagnostic Laboratory Network where standards for result interpretation are agreed upon by all members, and interlaboratory testing is done to maintain confidence in the assay's performance across a range of unique testing parameters (Blehert and Lorch, 2021). Second, the WNS case definition was updated to reflect the uncertainty inherent in testing samples at or ahead of the leading edge of the invasion front where pathogen loads are low and results may be more variable (Langwig and others, 2015b, c; Mosher and others, 2019; WNS DxWG, 2021). Third, this diagnostic laboratory handbook has been created to capture the best practices for sample collection, storage, and processing agreed upon by the WNS Diagnostic Laboratory Network and to serve as a reference for other laboratories wishing to join the network.

Standardization Versus Harmonization

Laboratory networks require a coordination framework to support uniform approaches to infectious disease diagnostic testing among multiple laboratories. Consistency can be achieved through harmonization or standardization of diagnostic methods and guidelines for interpreting results.

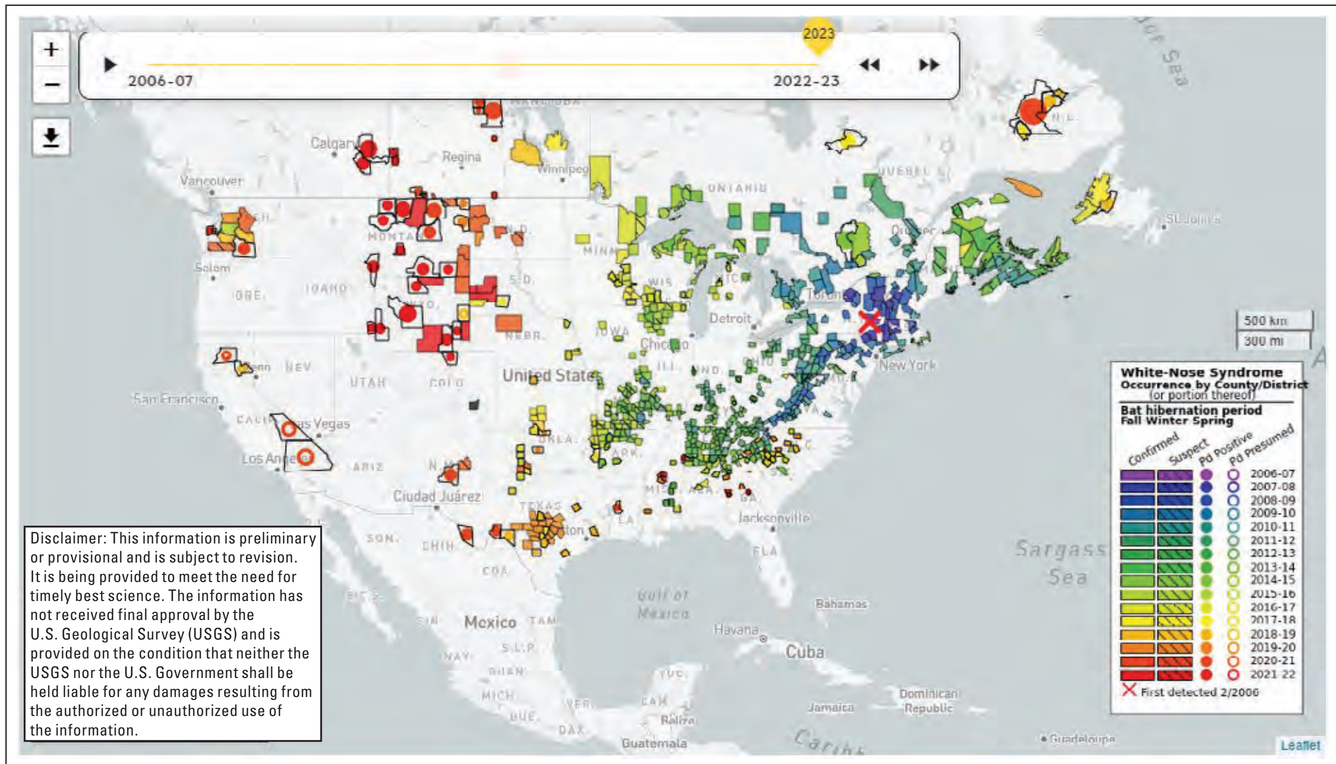


Figure 2. Progressive spread of *Pseudogymnoascus destructans* fungus and white-nose syndrome disease from the first documented outbreak in 2007 to April 28, 2023.

Standardization is a method for achieving equivalency of results from different laboratories by requiring that all tests be calibrated in a manner that can be traced to a known reference material or measurement protocol—typically defined using the International System of Units (American Association of Clinical Chemistry, 2015; Tate and Myers, 2016; Vesper and others, 2016). In human medicine, standardized reference materials and methods are subject to a rigorous certification process and extensive oversight as required by a regulatory body such as the National Institute of Standards and Technology or the WHO International Reference Preparation (Armbruster and Miller, 2007; Vesper and others, 2016). Harmonization, on the other hand, does not depend upon tracing results to the International System of Units, but instead ensures that diagnostic testing performed in different laboratories, potentially using different methods, will yield equivalent results that can be meaningfully compared to an agreed-upon reference material or measure (Vesper and others, 2016).

A governance structure is required to support uniform approaches to test for infectious diseases by different laboratories, and this structure can be either formal or voluntary. The USDA National Animal Health Laboratory Network (NAHLN) is an example of a formal network that coordinates and standardizes the work of all U.S. organizations providing disease surveillance and testing services relevant to domestic animal production. Within the NAHLN network, consistency is achieved through mandating the use of standardized equipment and controlled test reagents, requiring regular proficiency testing of laboratory staff, developing strict quality control and biosecurity systems, and reserving the right of a designated reference laboratory to make the final diagnostic decision in situations involving conflicting or uncertain results (Animal and Plant Health Inspection Service [APHIS] and National Institute of Food and Agriculture, 2012; Gosser and Morehouse, 1998). Alternatively, the WHO provides proficiency tests for member nation laboratories in an effort to estimate performance metrics while preserving each member's sovereignty in terms of protocol selection and use. This model is congruent with a growing call within wildlife disease diagnostics in the United States to harmonize results and reporting standards across disparate methodologies and laboratories (Blehert and Lorch, 2021). Successful harmonization minimizes uncertainty of diagnostic test results such that management agencies can confidently make evidence-based decisions amidst these differences (Sleeman and others, 2019).

Purpose and Scope

This handbook collates and documents best management practices for collecting, storing, and testing samples for *Pd* by qPCR. It is intended to provide guidance for laboratories participating in, or wishing to join, the WNS Diagnostic

Laboratory Network and to facilitate provision of accurate and consistent diagnostic information to land and wildlife managers. It is not intended to be exhaustive in terms of all aspects of wildlife disease surveillance and diagnostics (for example, statistical sampling design, safe capture and handling of live animals, development of new assays, and so on) as much has been written elsewhere on many of these topics (see box 2). In addition, individual laboratory protocols or shared working group documents (such as case definitions) that may be subject to updates or changes with time have also been omitted to extend the longevity of this handbook. Instead, where appropriate, references and links to additional resources are included.

The WNS Laboratory Network includes multiple laboratories from the United States and Canada (fig. 3) that have contributed protocols, performance metrics, and input into this handbook (a complete list of participating laboratories, along with appropriate contact information, can be found on the WNS Response Team website: https://www.whitenosesyndrome.org/). Some of these laboratories participate in annual WNS National Response surveillance efforts by processing samples collected using prepared sampling kits. Other laboratories receive samples through independent research projects or from State and regional submissions. This handbook harmonizes WNS diagnostic testing by documenting variations in methodologies for sample collection, storage, and processing and by outlining procedures for interpreting results to ensure consistency despite noted differences.

National White Nose Syndrome Response Team logo; used with permission.

Box 2. Additional Resources for Wildlife Disease Sampling

Much has been written elsewhere on these and other points related to pathogen sample collection in free-ranging wild animals. For additional information related to wildlife sample collection, please refer to the following resources:

- Franson and others (2015), specifically the following chapters:
  - Chapter B3: Mortality Investigation
  - Chapter C3: Recording and Submitting Specimen History Data
  - Chapter C4: Wildlife Specimen Collection, Preservation and Shipment, and
  - Chapter C7: Special Considerations for Specimen Collections that may be Involved in Law Enforcement Cases
- U.S. Department of Agriculture:
  - Animal Welfare Information Center website (National Agricultural Library, 2022)
  - “Research with Free-Living Wild Animals in Their Natural Habitat and the Animal Welfare Act” (Animal and Plant Health Inspection Service, 2022)
- World Organization for Animal Health (WOAH) National Focal Points for Wildlife, training manuals:
  - Training manual on wildlife diseases and surveillance (WOAH, 2010)
  - Training manual on surveillance and international reporting of diseases in wild animals (WOAH, 2015)
  - Training manual on wildlife disease outbreak investigations (WOAH, 2017)
- National Academies of Sciences, Engineering, and Medicine workshop proceedings on animal welfare challenges in research and education on wildlife, non-model animal species and biodiversity (National Academies of Sciences, Engineering, and Medicine, 2022)

U.S. Fish and Wildlife Service

A National Plan for Assisting States, Federal Agencies, and Tribes in Managing White-Nose Syndrome in Bats

May 2011

Marvin Moriarty/USFWS

Bat affected by white-nose syndrome

National white-nose syndrome response plan (U.S. Fish and Wildlife Service, 2011).

Map shape downloaded from Wikimedia Commons under license CC-BY-SA-2. Contributers: Lokal Profil, Alan Rokefeller, Lommes, Fluffy89502 and Ponderosapine210.
https://commons.wikimedia.org/wiki/File:North_America_second_level_political_division_2.svg

Figure 3. White-Nose Syndrome Diagnostic Laboratory Network members in 2021.

Principles of Wildlife Disease Sampling with Additional Resources

Accurate and reliable diagnostics begin with proper sample collection. Regardless of whether specimens are from humans, companion animals, livestock, or free-ranging wildlife, good sampling requires adherence to established protocols to guarantee the sample type is appropriate for the anticipated test, to minimize risk of contamination, to maintain sample integrity from collection to processing or storage, and to ensure safety of the collector and the individual being sampled. When test results are used to make broader statistical inferences about pathogen prevalence and distribution, disease dynamics, or other epidemiological parameters, sampling design also plays a critical role in obtaining useful information and should include (in consultation with a statistician if necessary) a thorough assessment of surveillance objectives, an understanding of the population targeted for surveillance, and statistical power.

When sampling for pathogens in wildlife populations, each of the above considerations involves complicating factors. These include, but are not limited to, difficulty collecting high-quality samples from live animals or diagnostic methods dependent upon sample types that can only be collected postmortem; challenging field conditions (such as extreme temperatures or remote locations) that can negatively affect sample integrity before its arrival in the laboratory; and risks of handling wildlife including stress or physical harm to the animal and injury to the handler. Additionally, field personnel should be aware of any potential role they may inadvertently play in spreading pathogens from one location or population to another through contaminated clothing, shoes, or equipment. Many of these concerns can be mitigated through advanced planning before starting field work to identify appropriate sample collection tools and sample-storage strategies to maintain cold chains. Review of methods for animal capture and restraint by the Institutional Animal Care and Use Committee will ensure compliance with statutes and regulations governing animal welfare. Consultation with the Institutional Biosafety Committee will ensure correct use of personal protective equipment (PPE) and adherence to recommended decontamination guidelines for clothing and equipment if moving between sites.

Sampling Considerations for *Pseudogymnoascus destructans*

It is important, in any surveillance program, to clearly define sampling objectives. In wildlife disease surveillance, limited access to various sample types and availability of diagnostic assays may create additional challenges for achieving surveillance objectives such as determining disease status of an individual animal or population. To maximize the actionable data available to managers, it is important for everyone involved in a wildlife disease surveillance project (including biologists, statisticians, technicians, and managers) to clearly communicate the questions they are asking, to understand how sample collection and testing procedures may affect the ability to answer those questions, and to recognize limitations and uncertainty inherent in any wildlife disease sampling program, including when an individual diagnostic result can and cannot answer the posed question (Mosher and others, 2019).

When defining diagnostic objectives, understanding the distinction between testing for a disease or disease process and testing for a pathogen or disease agent is important. The collection and testing of some sample types may be able to answer questions related to pathogen presence or distribution but unable to definitively answer questions related to disease manifestation or processes. As a result, the ability or inability to collect certain types of samples will affect the type of information that can be learned. Histopathology is considered the gold standard for diagnosing the disease WNS, but this technique requires whole carcasses or adequate skin biopsy samples and must be performed by a trained veterinary pathologist (fig. 4; Meteyer and others, 2009). Observed signs of the disease, in conjunction with the positive identification of *Pd* by qPCR or fungal culture and sequencing, are enough to designate a sample as suspect for WNS, but histopathologic analysis after necropsy or collection of a wing-skin biopsy is still required for definitive confirmation (Turner and others, 2014; WNS DxWG, 2021). This requirement poses limitations to disease surveillance efforts because managers may not have access to bats or may wish to avoid prolonged handling required for biopsy collection. For this reason, most organized surveillance samples collected to date are for the purpose of identifying the presence of the causative fungus, *Pd*.

Distinguishing between disease and pathogen surveillance is important because not all bat species are equally susceptible to developing WNS, and some tolerate colonization by the fungus without developing the disease (Zukal and others, 2016; Langwig and others, 2016). Our current understanding of the *Pd*/WNS system indicates that the detection of the pathogen on susceptible species in a new geographic area is a predictor of disease emergence (Langwig and others, 2015a, b; Hoyt and others, 2020). As both *Pd* and WNS continue to expand in geographic distribution across North America, however, changing environmental and climatic conditions, along with variations in species assemblages or roosting behaviors, may cause unexpected changes in patterns of fungal growth, transmission, or other biological and epidemiological aspects (Hayman and others, 2016; Lilley and others, 2018). If the surveillance objective is to detect WNS disease in new bat populations, sampling design may need to be adjusted to account for uncertainty related to pathogen presence and disease progression in novel environments.

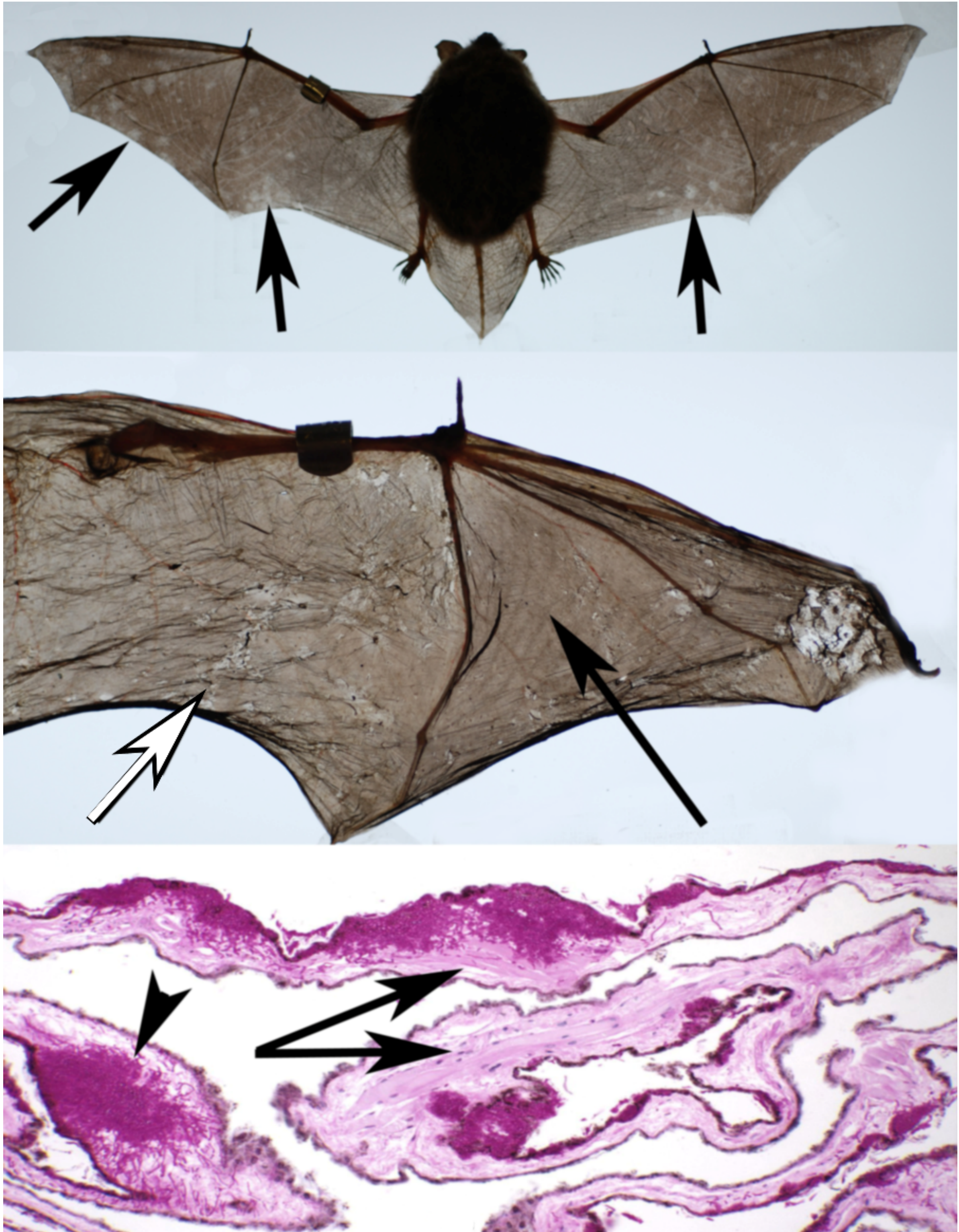


Figure 4. Examples of white-nose syndrome and *Pseudogymnoascus destructans* in bats. Photographs by Carol Uphoff Meteyer (U.S. Geological Survey; Cryan and others, 2010). *A*, backlit little brown bat positive for white-nose syndrome. *B*, backlit wing of a little brown bat positive for white-nose syndrome. *C,* wing membrane histology showing infection with *Pseudogymnoascus destructans.*

Biosecurity

Adequate biosecurity should be a top priority for any researcher collecting *Pd* samples in the field. Appropriate use of PPE is critical to maintaining the health and safety of bats and personnel, as well as limiting the potential spread of pathogens to new areas. The fungus is environmentally stable and can persist in sediment and on cave substrate even during summer months when bat populations may be reduced in number or absent (Ballmann and others, 2017; Verant and others, 2018). In addition, *Pd* spores can be carried outside of the original cave environment by nonhibernating bats during the summer (Carpenter and others, 2016; Huebschman and others, 2019) or by humans via contaminated research equipment or recreational caving equipment (Ballmann and others, 2017; Zhelyazkova and others, 2020), and the fungus remains viable on various surfaces and at elevated temperature for anywhere from days to months (Hoyt and others, 2015; Campbell and others, 2020; Zhelyazkova and others, 2020). Because of this, extra precaution is warranted during sample collection to prevent spread of the pathogen to other pathogen-free sites. Risk assessments, biosecurity recommendations, and decontamination protocols for wildlife disease (and specifically *Pd*) field sampling are available from numerous sources and should always be followed as closely as possible to minimize risk of harm to personnel, targeted animals, and sensitive ecosystems (table 1).

Collection Methods

Multiple sample collection methods exist for *Pd* and WNS surveillance. These methods differ in their degree of invasiveness (direct handling of bats) and the type of diagnostic information they provide (fig. 5). Collection practices also typically take into consideration factors such as site accessibility, availability of trained personnel, or the time of year that sampling takes place. Methods are described broadly in the following sections, and resources for additional details on sampling methodology are provided in box 3.

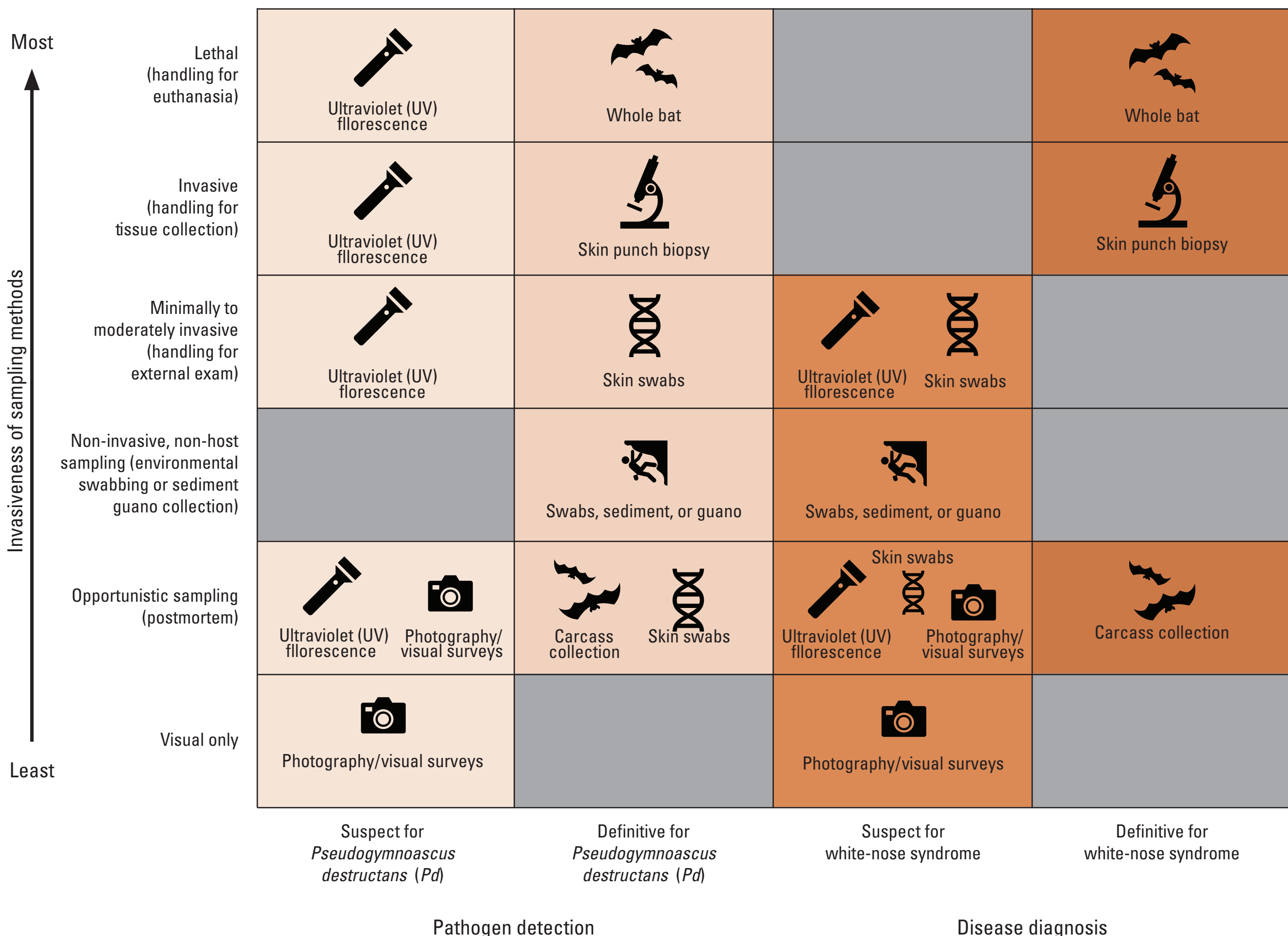
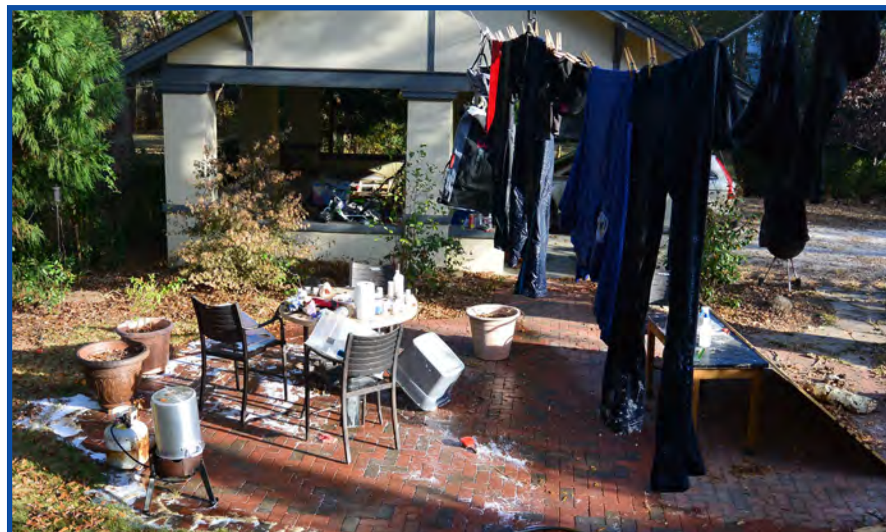


Figure 5. Diagnostic information and degree of invasiveness for various *Pseudogymnoascus destructans* sample collection methods.

Table 1. Examples of various resources for biosecurity information and decontamination recommendations related to wildlife disease sampling in general and *Pseudogymnoascus destructans* sampling in particular.

[SARS-CoV-2, novel β-coronavirus; USGS, U.S. Geological Survey; USFWS, U.S. Fish and Wildlife Service; WNS, white-nose syndrome; *Pd*, *Pseudogymnoascus destructans*; WOAH, World Organisation for Animal Health]

Source	Description	Link (accessed May 2023)
Centers for Disease Control and Prevention	Reducing the risk of SARS-CoV-2 spreading between people and wildlife	https://www.cdc.gov/healthypets/covid-19/wildlife.html
International Union for Conservation of Nature (IUCN), Bat Specialist Group	Recommended strategy for researchers to reduce the risk of transmission of SARS-CoV-2 from humans to bats	https://www.iucnbsg.org/bsg-publications.html
USGS and USFWS	Assessing the risks posed by SARS-CoV-2 in and via North American bats	https://doi.org/10.3133/ofr20201060
National WNS Response Team Disease Management Working Group	Current guidelines for *Pd*decontamination information (equipment and personnel)	https://www.whitenosesyndrome.org/static-page/decontamination-information
National WNS Response Team Disease Management Working Group	White-nose syndrome show cave guidance: Recommended practices to reduce risks of people spreading the fungus *Pd*	https://www.whitenosesyndrome.org/mmedia-education/show-cave-guidance
USGS, USFWS, and National Park Service	Safe work practices for working with wildlife	https://doi.org/10.3133/tm15C2
American Association of Zoo Veterinarians Animal Health and Welfare Committee	Infectious disease manual: Infectious diseases of concern to captive and free ranging wildlife in North America	https://cdn.ymaws.com/www.aazv.org/resource/resmgr/idm/idm_updated_march_2020.pdf
WOAH Technical Disease Cards for non-WOAH -Listed Diseases in Wildlife	*Pseudogymnoascus destructans*in bats (white-nose syndrome) (Infection with)	https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/newcards/Pseudogymnoascus_destructans_in_bats_(White-nose_syndrome)(Infection_with).pdf
Canadian Wildlife Health Cooperative	Canadian national white-nose syndrome decontamination protocol for entering bat hibernacula	https://s3.us-west-2.amazonaws.com/prod-is-cms-assets/wns/prod/2ef38950-795a-11e8-b1a2-85f666b81922-WNS_Decontamination_Protocol-Mar2017.pdf
Canadian Wildlife Health Cooperative	Recommendations for WNS decontamination during summer activities	http://www.cwhc-rcsf.ca/docs/miscellaneous/Recommendations%20for%20WNS%20decontamination%20during%20summer%20activities.pdf



“National White-Noise Syndrome Response Decontamination Protocol,” revised 2020 by the Disease Management Working Group (http://www.whitenosesyndrome.org/static-page/decontamination-information).

Box 3. To Learn More About Sampling

More detailed information related to sampling for *Pseudogymnoascus destructans* and white-nose syndrome are available from various sources:

- The U.S. Geological Survey National Wildlife Health Center maintains an annually updated document detailing various sample types and collection procedures for detecting *Pseudogymnoascus destructans* and white-nose syndrome and for storing and shipping samples: https://www.usgs.gov/centers/nwhc/science/white-nose-syndrome-surveillance?qt-science_center_objects=0#qt-science_center_objects.
- The Canadian Wildlife Health Cooperative provides instructions for collecting and submitting bat skin swabs: http://www.cwhc-rcsf.ca/docs/How_to_survey_for_WNS.pdf.
- The White-Nose Syndrome Diagnostic Working Group has links to instructional videos covering several different sample collection procedures (videos follow National Wildlife Health Center protocols): https://www.whitenosesyndrome.org/working-group/surveillance-and-diagnostics.

Methods described may differ slightly. Before collecting samples, check with the processing laboratory to see if they have specific instructions or recommendations. Any sampling method (lethal or nonlethal) that requires handling of live bats should be reviewed by a relevant Institutional Animal Care and Use Committee or other oversight body, and implementing these methods further requires proper training and use of personal protective equipment to minimize risk of pathogen exposure to the handler and the animal (Taylor and Buttke, 2020; Runge and others, 2020). In addition, collectors should ensure they have all appropriate State, Provincial, and Federal collection permits before sampling, particularly when threatened or endangered species may be involved.

Noninvasive Sampling (No Animal Handling Required)

In some cases, clinical signs consistent with WNS can be identified visually (Janicki and others, 2015). Studies indicate that photography is an effective tool for bat population surveys and has the additional advantage of allowing follow-up inspection for visible signs of fungal growth on individuals or colonies (Meretsky and others, 2010; Puechmaille and others, 2011). Although appealing as a low-cost alternative to more invasive sampling methods, efficacy of visual surveys for WNS can be influenced by fungal load or the time of year the survey is done (Janicki and others, 2015). Bats can also host other nonlethal species of fungi that are visibly similar to *Pd*, making fungal growth alone insufficient for definitive diagnosis of WNS (Lorch and others 2015). Therefore, sample collection methods that allow for definitive *Pd* detection or disease diagnosis (fig. 5) are recommended when the presence of *Pd* is suspected at a site or in a colony of previously unknown *Pd*/WNS status.

Sediment, substrate swabs, and guano are noninvasive sample types that can be collected from bat hibernacula or roost sites (fig. 6). These sample matrices can be tested for the presence of *Pd* DNA and may additionally contain viable fungus for culture and isolation (Lindner and others, 2011; Lorch and others, 2013a, b; Ballmann and others, 2017; Campbell and others, 2020). After the first invasion, the persistence and amount of *Pd* in the environment from one season to the next can increase with population prevalence, making environmental samples potentially important indicators of fungal load within a bat population or associated reservoir for infection or dispersal (Langwig and others, 2015a, b; Ballmann and others, 2017; Hoyt and others, 2020). Noninvasive samples are also suitable options for surveillance when capturing or handling bats is impractical, impossible, or constitutes an unreasonable risk to either the animal or the handler (Runge and others, 2020).

Despite these advantages, there are also several challenges to getting reliable diagnostic information from these sample types. Soil, sediment, or guano all contain naturally occurring substances that inhibit qPCR reactions, as well as a wide variety of other microorganisms that make culturing *Pd* difficult (Lorch and others, 2013a, b). In addition, several studies documented a difference of as much as 1 year between initial detections of *Pd* on bats at a hibernaculum and subsequent environmental detections at the site (Langwig and others, 2015b; Verant and others, 2018). These limitations may mean that other sample types are better suited to early detection of *Pd* in new areas.

Minimally to Moderately Invasive Sampling (Animal Handling Required for External Examination)

When capturing and handling bats is feasible, other sampling methods are available to assess for *Pd* and WNS. Wing lesions diagnostic for WNS will exhibit subtle yellow-orange fluorescence when illuminated with ultraviolet (UV) light within the spectral region of 368 to 385 nanometers (fig. 7; Turner and others, 2014). Studies have shown strong agreement between UV fluorescence and histopathology for identifying WNS, making UV illumination a useful, nonlethal

Figure 6. Guano collection for *Pseudogymnoascus destructans* testing at Governor Dodge State Park, Dodgeville, Wisconsin. Photographs by Kyle George, U.S. Geological Survey.

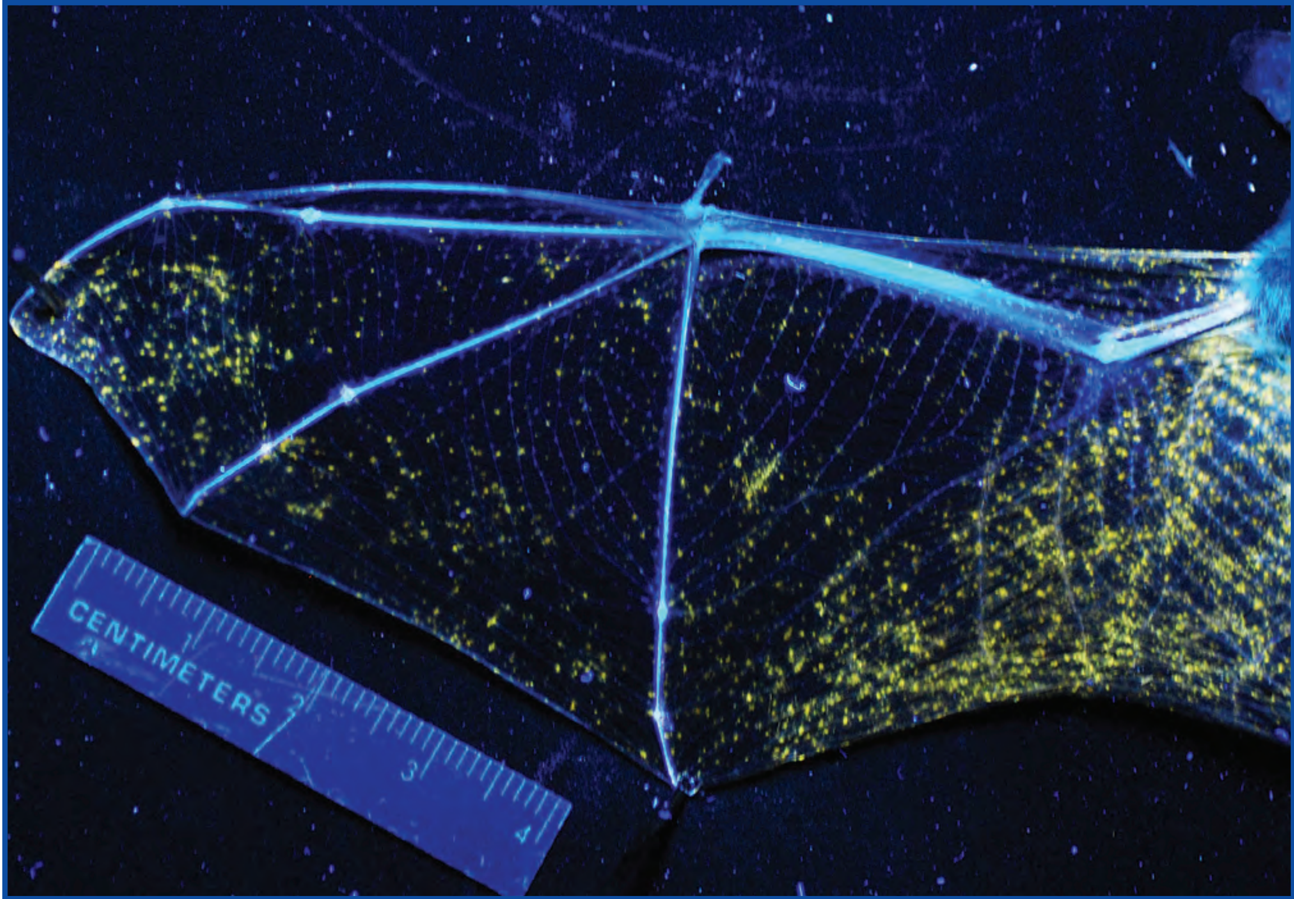


Figure 7. Characteristic yellow-orange fluorescence of *Pseudogymnoascus destructans* fungal lesions on wing of infected bat under ultraviolet light. Photograph from Turner and others (2014); used with permission.

screening tool to assess the presence and severity of disease (Turner and others, 2014; McGuire and others, 2016; Pikula and others, 2017). Additionally, by providing the ability to screen for WNS-associated lesions, UV fluorescence facilitates targeted, nonlethal collection of skin biopsy samples, which may be preferable to euthanasia (Pikula and others, 2017); however, UV fluorescence has several limitations as a diagnostic method. First, the tool is only useful for detecting fungal invasion of the skin and does not perform as well as qPCR for early detection of the fungus itself (McGuire and others 2016). The tool also is not diagnostically definitive for WNS, particularly during nonhibernal times of the year when fluorescence specific to WNS decreases as lesions heal (Pikula and others, 2017; Ballmann and others, 2017). For these reasons, fluorescence-based screening for lesions characteristic of WNS should be followed up with other testing methods to ensure an accurate disease diagnosis.

Collecting samples of epidermal microflora using a sterile swab is a common method for fungal pathogen surveillance in wildlife (Hyatt and others, 2007; Langwig and others, 2015a; Allender and others, 2016; McGuire and others, 2016). Bat skin swabs collected for testing are analyzed for the presence of *Pd* by molecular qPCR assay (fig. 8; Muller and others, 2013). This method of fungal detection has outperformed other diagnostic methods such as UV fluorescence and histology in the early stages of infection, making skin swabbing a good surveillance method for early detection in hibernating bats (McGuire and others, 2016; Ballmann and others, 2017). Although protocols deployed by different entities to collect and screen samples for *Pd* by qPCR may differ in certain details (for example, recommended number of swab passes per animal or use of a preservative for sample storage; box 3), general guidelines are consistent. How variations in methodology may affect diagnostic outcomes is not yet well characterized.

When captured bats are restrained in separate holding bags before swabbing, collecting fresh guano may also be possible. Results of a recent study indicate that fresh guano may be a better sample than skin swabs for detecting *Pd* during the summer when bats are more likely to ingest the fungus by

Figure 8. *Pseudogymnoascus destructans* surveillance sample collection via epidermal swabbing. Photograph by Kyle George, U.S. Geological Survey.

grooming, subsequently decreasing their external fungal load (Ballmann and others, 2017). This study points to the benefits of collecting multiple sample types in tandem to obtain a more detailed picture of invasion dynamics throughout different sites and times of the year (Langwig and others, 2015a; Ballmann and others, 2017; Verant and others, 2018).

Invasive Sampling (Animal Handling Required for Tissue Collection)

Although using the UV fluorescence technique and collecting skin swabs can involve capturing and handling individual bats, these techniques are not expected to inflict discomfort on the animal when done correctly. Tissue collection via punch biopsy is a collection method for *Pd* and WNS surveillance that may cause stress or pain to the bat being handled and should therefore only be done by individuals properly trained to perform the technique (Pikula and others, 2017). Skin biopsies are commonly collected for histopathology analysis to confirm the disease WNS or as a source of host genetic material that can be used to evaluate individual and species genome characteristics (Turner and others, 2014; Lilley and others, 2019). As mentioned previously, using UV fluorescence to guide the biopsy selection site for disease confirmation can improve sample quality and thus diagnostic value (Turner and others, 2014; Pikula and others, 2017).

Lethal Sampling (Animal Euthanized and Collected for Diagnostic Purposes)

Developing nonlethal collection methods for WNS and *Pd* testing has resulted in less frequent need to euthanize bats for sampling (Turner and others, 2014; McGuire and others, 2016). When euthanasia is appropriate or unavoidable, the whole carcass can be collected for further testing. Intact, fresh carcasses can have a high diagnostic value because a variety of samples and tissue types can be collected to support multiple testing methods (such as histopathology, molecular assays, culture) or preservation for future study (Keller and others, 2021). Regardless of whether euthanasia is an intended outcome, sampling protocols involving live animals should always be reviewed by the relevant Institutional Animal Care and Use Committee, which requires anticipating and describing potential euthanasia techniques, should an animal be lethally injured during capture. Lethal sampling and euthanasia should always be done by trained professionals following the applicable State, Provincial, and Federal guidelines for capture and handling of bats, as well as the American Veterinary Medical Association guidelines for humane euthanasia of wild mammals (https://olaw.nih.gov/policies-laws/avma-guidelines-2020.htm).

Opportunistic Sampling (Animal Collected for Purposes Other than White-Nose Syndrome Diagnostic Testing)

Collecting data from animals found dead or collected for other purposes (for example, accidental death, roadkill, or euthanasia of nuisance animals) can create challenges for predictive modeling or for making statistical inferences (Nusser and others, 2008; Davis and others, 2019); nevertheless, opportunistic collection of samples from wildlife is common, can yield useful information about pathogens, and can be part of a robust surveillance program. For WNS, sources of opportunistic samples include State public health and hygiene laboratories that test bats for rabies, passive collection of carcasses from mortality events, removal of nuisance individuals by wildlife control operations, or wildlife rehabilitators (Lorch and others, 2016; Darling and others, 2017).

Laboratory Biosecurity and Quality Management Systems

Laboratory biosafety levels (BSLs) indicate the degree of containment and security required for the types of agents handled and work performed in a particular space (WHO, 2004; Meechan and Potts, 2020). International guidelines for these levels are provided by the WHO and WOAH, but specific regulations, standards, and compliance metrics are set at the national level (WHO, 2020). In the United States, BSL designations are determined by the CDC and USDA Agricultural Research Services, whereas in Canada, the Public Health Agency of Canada (PHAC) and Canadian Food Inspection Agency define containment levels to regulate the handling of human and animal pathogens, respectively. In each country, these levels correspond to various requirements related to personnel training, physical space, ventilation and airflow, required equipment, and security barriers (PHAC, 2015; Meechan and Potts, 2020). Specific work is classified based on the assessed risk of the agent and the activity to individuals (human or animal), the environment, and the community (table 2; PHAC, 2015; WOAH, 2018a; Meechan and Potts, 2020; WHO, 2020).

Although *Pd* is not known to infect humans, the fungus is designated a BSL-2 agent because of its pathogenicity to bats and subsequent population effects (Blehert and Lorch, 2021); therefore, diagnostic or research work with *Pd* is expected to be done in a space designated as BSL-2/CL-2 or higher (USFWS, 2011; Inter-agency White-Nose Syndrome Committee, 2015). This includes precautions such as providing personnel with specialized training on handling infectious materials, doing work inside of a biological safety cabinet (BSC) to contain aerosolized spores, autoclaving all biomedical waste, following shipping guidelines for dangerous goods when transferring *Pd* to other BSL-2 facilities, and asking individuals working with the fungus to observe a voluntary 7-day quarantine during which they will not enter caves or other sites where bats are known to congregate (USGS, 2015; Blehert and Lorch, 2021). Ventilation and airflow requirements are not specified for BSL-2 laboratories (WHO, 2004; Meechan and Potts, 2020); however, good practice for laboratories doing PCR is to ensure that areas where amplicons are generated or analyzed maintain a slightly negative pressure so air currents are pulled into the space rather than flowing out and potentially contaminating other laboratory areas (fig. 9;

Table 2. General descriptions of the four biosafety/containment laboratory levels in the United States and Canada including acceptable risk levels and example agents for each.

[BSL, biosafety level; CL, containment laboratory]

Biosafety and containment level	Criteria	Example agents
BSL–1/CL–1	Laboratory suitable for handling and storage of biologic material that is well characterized and is incapable or unlikely to cause disease in otherwise healthy humans or animals and poses low risk to the environment.	Nonpathogenic strains of *Escherichia coli.*
BSL–2/CL–2	Laboratory suitable for handling and storage of biologic material known to cause disease in humans or animals of variable severity and moderate risk to the environment.	Hepatitis B, *Salmonella, Pseudogymnoascus destructans, Toxoplasma.*
BSL–3/CL–3	Laboratory suitable for handling and storage of biologic material that is capable of respiratory transmission, that may cause severe or lethal disease in humans and animals, or that poses a substantial risk to community and (or) the environment.	*Coxiella burnetii, Burkholderia* spp., Highly pathogenic avian influenza virus.
BSL–4/CL–4	Laboratory suitable for handling and storage of biologic material that pose high risk of severe and fatal disease to individuals or community, that pose a high risk of aerosol transmission, and for which there are no effective treatments.	Marburg virus, Ebola virus, Nipah virus.

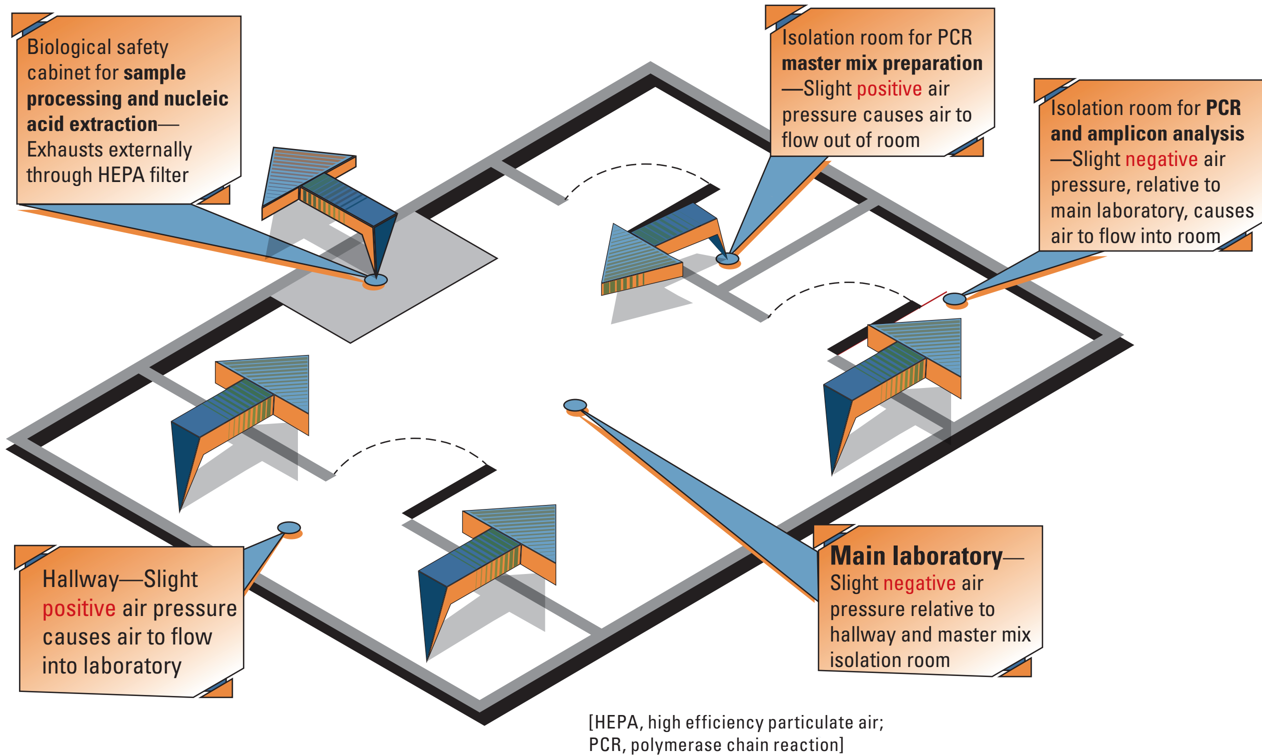


Figure 9. An example of a polymerase chain reaction laboratory with airflow regulated by areas of differing air pressure.

Dieffenbach and Dveksler, 2003). Members of the WNS Diagnostic Laboratory Network should be designated as, at minimum, BSL-2 (USGS, 2015; Meechan and Potts, 2020) and are encouraged to consider the physical layout of their space in a way that uses unidirectional airflow from areas least likely to generate contamination materials to those most likely (see “Minimizing Contamination” section for more information on unidirectional workflow).

Regardless of whether a laboratory is State, Provincial, Federal, or academically affiliated, the documentation and implementation of a quality management system (QMS) is paramount to the integrity of that facilities’ diagnostic results (International Organization for Standardization [ISO], 2015; WOAH, 2018f). Clear procedures to guide appropriate workflow, to minimize potential for contamination, and to describe proper storage, handling, and processing of samples serve to increase certainty in laboratory results and to increase confidence in end users with decision-making authority. Clear workflow documentation is particularly important for the performance of real-time qPCR assays, which have gained popularity because of their fast turnaround time for results, relative ease of use, and ability to detect very small amounts of target genetic material (Kralik and Ricchi, 2017); as one microbiologist lamented, “it is remarkably difficult to make a reaction fail completely but alarmingly simple to produce poor quality data” (Johnson and others, 2013, p. 3). QMS provides the framework to establish and control work processes to ensure data integrity.

The American Association of Veterinary Laboratory Diagnosticians (AAVLD) defines a QMS as “a combination of organizational structure, resources, people, documents, and activities designed to ensure a consistent product and services that meet the needs of an organization’s clients” (AAVLD, 2017). A QMS can be established as part of a formal institutional accreditation via an internal accountability process (for example, U.S. Geological Survey Instructional Memorandum OSQI 2018–01, available online: https://www.usgs.gov/survey-manual/im-osqi-2018-01-quality-management-system-usgs-laboratories), or through consensus by a voluntary laboratory network. The WNS Diagnostic Laboratory Network includes accredited and nonaccredited member laboratories, but all participating institutions should follow QMS principles that, at minimum, include the following:

- standard operating procedures to document data related to sample collection and identification of samples throughout the workflow, sample handling and storage processes for the appropriate biosecurity designation, diagnostic testing procedures, and equipment maintenance and calibration;
- tracking systems for the creation, storage, and performance of controls; and
- a data management plan for the dissemination and storage of results.

Other potential components of a QMS may include the following:

- employee training and (as appropriate) proficiency testing records, and
- corrective action reports to document and diagnose unexpected or erroneous results or incidents related to equipment failure or personnel error.

Sharing protocols among laboratories provides context when questions or issues arise from testing, and collaboration within the network provides support for troubleshooting discrepancies.

Controls

Using positive and negative controls is fundamental to sound diagnostic testing and can be implemented throughout different points in the process to gauge if procedures and reactions are happening as expected (positive controls), to ensure that cross-contamination has not happened during processing (negative controls), and to assess for background signal from reaction reagents (table 3; Burd, 2010). A rigid standard does not exist for determining how molecular assay controls need to perform across tests and laboratories; however, there is general agreement that certain types of controls should be included. For qPCR assays, controls (positive and negative) are expected for the nucleic acid extraction and amplification processes. If contamination in the field during sample collection or maintaining the integrity of a sample type is of concern, sampling controls (known positive or negative samples that are subject to identical handling and storage conditions as diagnostic samples) may also be recommended (Laurin and others, 2018).

Traditionally, positive amplification controls have been developed in several ways. Nucleic acid can be extracted directly from the target organism and diluted in a neutral matrix to a known concentration, amplified target material can be quantified and purified, or target sequences can be cloned into a plasmid. The pros and cons of these more traditional methods have been detailed elsewhere (Dhanasekaran and others, 2010). More recently, synthetic oligonucleotides (such as gBlocks) have become an appealing alternative for several reasons, one of which is allowing the user to insert unique markers into the sequence to differentiate the control from the natural target (Conte and others, 2018). Presence of this marker helps rule out cross contamination between the control and a diagnostic sample. Additionally, synthetic oligonucleotides are inexpensive and easy to obtain, whereas other methods of generating amplification controls require in-house labor and create risk for contaminating laboratory space with genetic material targeted by the diagnostic assay (Dhanasekaran and others, 2010; Conte and others, 2018).

In addition to separate amplification controls, some laboratories may also choose to include an exogenous internal positive control (IPC) within the qPCR reaction to evaluate for the potential presence of inhibitory substances (Hoorfar and others 2004; Das and others 2009; Burd 2010). These controls consist of a nontarget DNA sequence that is multiplexed along with the target in the same reaction and, when the reaction performs as expected, should always amplify (Hoorfar and others 2004). Therefore, amplification of the IPC along with no amplification of the target indicates a reliable negative result, whereas failure of the IPC to amplify along with the target indicates a failure of the PCR reaction (Hoorfar and others, 2004; Burd, 2010). Diagnostic samples collected from wildlife commonly come mixed with organic and inorganic substrates containing potentially inhibitory compounds that interfere with DNA amplification and other downstream analyses (Wilson, 1997; Das and others, 2009; Standish and others, 2018). Incorporating the use of an IPC can help prevent false negatives related to this inhibition (Hoorfar and others, 2004; Conraths and Schares, 2006). Within the WNS Diagnostic Laboratory Network, the use of an IPC is encouraged but not required.

Before performing qPCR for diagnostic purposes, nucleic acid must be extracted from the original sample material. Like the amplification process, this step also requires verification of expected performance, specifically related to cell lysis and nucleic acid recovery. Including controls is particularly critical with assays designed to detect fungal DNA because fungal cell walls can contain chitin, melanin, and other compounds that are difficult to break down using basic lysis chemicals such as proteolytic enzymes and detergents (Karakousis and others, 2006). Often, a combination of mechanical, chemical, and (or) enzymatic disruption procedures is necessary to break down fungal cell walls and recover DNA (Fredricks and others, 2005; Karakousis and others, 2006). To verify successful DNA extraction and purification, the best practice is to include a control that consists of the same target cellular material expected in the diagnostic samples and is spiked with or contains a known amount of target nucleic acid. This practice ensures the extraction control must go through the same lysis and nucleic acid recovery procedures as the test samples and can alert personnel to problems in the pre-qPCR sample processing phase. The highest level of assurance is achieved through using extraction controls that closely mimic or are identical in target organism and matrix (table 3; WOAH, 2018g).

For *Pd* qPCR testing, various positive controls are used by laboratories within the WNS Laboratory Network, and the acceptable value range for controls is not standardized among laboratories. Instead, each laboratory should have a predetermined, within-laboratory range that they use to gauge whether controls “pass” or “fail,” indicating a potential problem with the sample or procedure (fig. 10; Burd, 2010; WOAH, 2018c). WOAH’s Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (WOAH, 2018g) recommends using two amplification controls made with different amounts of starting target material: one “strong” positive control (in other words, low cycle threshold [Ct] value) and one “weak” (in other words, high Ct value). Successful amplification of both

Table 3. Summary of commonly used controls for *Pseudogymnoascus destructans* deoxyribonucleic acid extraction and quantitative polymerase chain reaction.

[An internal assay control is not included because of differences in workflow from the other four control types. For additional information, see Das and others (2009). DNA, deoxyribonucleic acid; *Pd, Pseudogymnoascus destructans;* PCR, polymerase chain reaction; TE, *tris*-ethylenediaminetetraacetic acid]

Control	Material and procedure	Purpose	Error being controlled
Positive amplification control (PAC)	gBlocks, plasmid DNA, or *Pd* genomic material in nuclease-free water or TE buffer; test along with samples	Verifies that PCR amplification performs as expected	Prevents false negatives (failure to detect the target when it is present in the original sample)
Negative amplification control (no template control [NTC])	Nuclease-free water; test along with samples	Detects potential contamination in PCR reagents, or carry-over contamination from samples or other controls	Prevents false positives (detecting the target when it is not present in the original sample)
Positive extraction control (PEC)	Neutral matrix spiked with known concentration of fungal conidia; extract and test along with samples	Verifies that extraction procedure performed as expected and nucleic acid recovery is sufficient for detection	Prevents false negatives (failure to detect the target when it is present in the original sample)
Negative extraction control (NEC)	Clean sample matrix; extract and test along with samples	Detects potential contamination in extraction reagents	Prevents false positives (detecting the target when it is not present in the original sample).

Result combination scenarios	Positive amplification control (PAC)	No-template control (NTC)	Positive extraction control (PEC)	Negative extraction control (NEC)	Interpretation	Recommended action
Combo 1	+	−	+	−	Controls and test performed as expected	Report results
Combo 2	I −	−	I −	−	Potential amplification problem due to inhibition, reagent quality issue, or technical error	Retest all samples
Combo 3	+	I +	+	− I +	Potential cross-contamination from PAC or sample, reagent quality issue, or technical error	Retest all samples
Combo 4	+	−	I −	−	Extraction performed sub-optimally or failed due to reagent quality or technical error	Repeat extraction from original material if possible, and retest all samples
Combo 5	+	−	+	I +	Potential cross-contamination during extraction from PEC or sample, reagent quality issue, or technical error	Repeat extraction from original material if possible, and retest all samples

EXPLANATION

+ **Positive**—Cycle threshold (Ct) within expected range

I **Inconclusive**—Cycle threshold (Ct) outside expected range

− **No amplification**—No cycle threshold (Ct)

Figure 10. Potential outcomes for amplification and extraction controls, the significance, and recommended actions for each scenario.

controls (which occur at the beginning and the end of the exponential growth phase of the curve, respectively) demonstrates reliable assay performance for field samples containing wide-ranging amounts of target nucleic acid.

No template controls or negative controls are added throughout the setup process and run alongside the diagnostic samples. Some laboratories may choose to include one set of negative controls that undergo every step of the process, from DNA extraction through qPCR processing (Burd, 2010). Others, however, may include a set of negative extraction controls as well as separate negative amplification controls to pinpoint the origins of potential contamination problems more precisely (Toohey-Kurth and others, 2020). Regardless, the WOAH recommends no less than 5 percent of reactions in each qPCR run consist of negative controls that are distributed randomly among the diagnostic samples (WOAH, 2018c). This number increases the probability that any cross contamination present will appear as amplification in at least one of the negative wells/tubes; however, randomly distributing the controls throughout the samples of each qPCR run can be cumbersome for high-throughput laboratories that use a standardized sample layout to streamline workflow. To achieve a compromise between efficiency and efficacy, negative controls can be spaced throughout the sample lineup in consistent locations (fig. 11) or in the last position(s) of the run to increase the probability of capturing potential contamination (Burd, 2010).

Minimizing Contamination

The proper use of controls can alert laboratories to contamination; however, minimizing that risk should be a priority in any diagnostic setting. With techniques such as qPCR that are highly sensitive, extra care must be taken to prevent false positives related to laboratory cross-contamination. Good laboratory practices related to space, equipment, and workflow can help minimize the possibility of contamination during sample processing (table 4; Conraths and Schares, 2006; Hopkins, 2008). Physical separation between workspaces in the laboratory, unidirectional workflows that control movement of personnel and equipment, and air flow controls that minimize aerosols are some of the fundamental strategies to minimize contamination in a molecular diagnostic laboratory (table 4; figs. 9 and 12; Hopkins, 2008). Additionally, most laboratories have systems in place for detecting and investigating potential contamination issues when suspect results arise.

The setup of a laboratory's physical space can aid or inhibit efforts to control contamination. The numerous steps involved in qPCR testing (including storing and processing samples, extracting nucleic acid, preparing qPCR master mix, thermocycling, analyzing results, and purifying PCR products for potential downstream applications such as sequencing) differ in terms of being considered "clean" or "dirty" based on the likelihood for result-altering contamination (fig. 12). Additionally, the space used to prepare positive extraction and qPCR controls should be taken into consideration. Any use or combination of reagents in the absence of sample material or positive control material can be considered "clean," whereas steps involving the manipulation of sample material or the production of amplicons is considered "dirty."

Areas where reagents are pipetted for extraction or where qPCR master mix is prepared should be as isolated as possible (in different rooms or opposite sides of the same room) from areas where samples or extraction eluates are manipulated. In laboratories that frequently perform qPCR for the same target organism, amplified product, known as amplicons, can accumulate in the area on and around the thermocycler, an occurrence called "amplicon buildup" (Persing, 1991; WOAH, 2018c). Overall, the large quantities of product generated during a qPCR experiment mean that contamination can happen

	1	2	3	4	5	6	7	8	9	10	11	12
A	DS	DS	DS	PAC std 1	DS	DS	DS	DS	NTC	DS	DS	DS
B	DS	DS	DS	NTC	DS	DS	DS	DS	NTC	DS	DS	DS
C	DS	DS	DS	NTC	DS	DS	DS	DS	NTC	DS	DS	DS
D	DS	DS	DS	NTC	DS	DS	DS	DS	NTC	DS	DS	DS
E	DS	DS	DS	NTC	DS	DS	DS	DS	NTC	DS	DS	DS
F	DS	DS	DS	NTC	DS	DS	DS	DS	NTC	DS	DS	DS
G	DS	DS	DS	NTC	DS	DS	DS	DS	NTC	DS	DS	DS
H	DS	DS	DS	PAC std 2	DS	DS	DS	DS	PAC std 3	DS	DS	DS

EXPLANATION

Diagnostic sample (DS)

Positive amplification control (PAC) three-point standard (std) curve

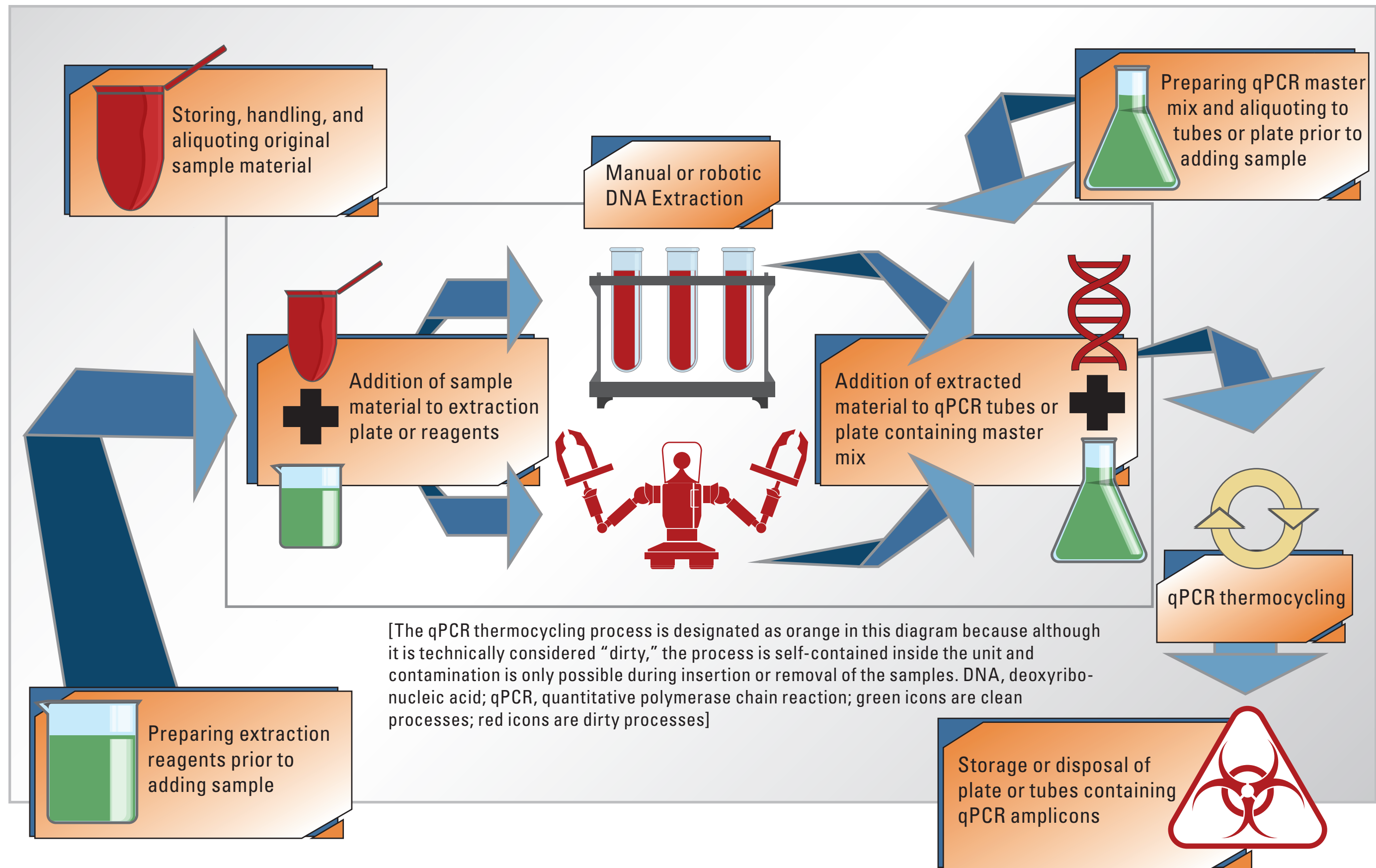
Negative/no template control (NTC)

Figure 11. Example quantitative polymerase chain reaction 96-well plate layout containing a 3-point standard curve as a positive amplification control, 13 negative/no template controls, and 80 diagnostic samples. Diagram by the Pathogen and Microbiome Institute at Northern Arizona University, a member of the White-Nose Syndrome Diagnostic Laboratory Network; used with permission.

Table 4. Summary of potential contamination sources throughout the quantitative polymerase chain reaction testing process and examples of mitigation strategies.

[BSC, biological safety cabinet; qPCR, quantitative polymerase chain reaction; NTC, no template controls]

Stage of process	Source of contamination	Mitigation strategy
Extraction	Positive control	● Add extraction control last. ● Pair with negative extraction control. ● Prepare single-use aliquots of positive controls.
	Other samples in batch	● Open only one sample at a time. ● Work inside a BSC to minimize aerosols. ● Change gloves frequently.
	Laboratory equipment or reagents	● Clean equipment and workspace before and after each use, using hospital-grade disinfectant (such as Unicide) or 10–20 percent diluted bleach followed by 95-percent ethanol. ● Perform extraction in dedicated area or BSC. ● Use dedicated pipettors and consumables. ● Institute regular environmental swabbing to identify potential sources of contamination. ● Include negative extraction control.
qPCR reaction setup and master mix aliquoting	Laboratory equipment or reagents	● Clean equipment and workspace before and after each use, using hospital-grade disinfectant (such as Unicide) or 10–20 percent diluted bleach followed by 95-percent ethanol. ● Use dedicated area or BSC for qPCR setup, with dedicated pipettors and consumables. ● Prepare single-use aliquots of reagents. ● Institute regular environmental swabbing to identify potential sources of contamination. ● Include NTC.
Addition of sample	Positive control	● Add amplification control last. ● Maintain dedicated pipettor for addition of control. ● Pair with NTC. ● Prepare single-use aliquots of positive controls.
	Other samples in batch	● If using tube-based extraction, open only one sample at a time. ● If using plate-based extraction and multichannel pipettes, discharge on the side of the well, just under the rim, to avoid splashes; work across the plate in one direction. ● Work inside a BSC to minimize aerosols. ● Change gloves frequently. ● Include NTC—distribute among samples or across plate.
	Laboratory equipment	● Clean equipment and workspace before and after each use, using hospital-grade disinfectant (such as Unicide) or 10–20 percent diluted bleach followed by 95-percent ethanol. ● Maintain dedicated locations and purposes for qPCR equipment. ● Ensure plates or tubes are sealed tightly before transporting to machine. ● Dispose of tested plates or tubes without disrupting seal. ● Institute regular environmental swabbing to identify potential sources of contamination.



[The qPCR thermocycling process is designated as orange in this diagram because although it is technically considered "dirty," the process is self-contained inside the unit and contamination is only possible during insertion or removal of the samples. DNA, deoxyribonucleic acid; qPCR, quantitative polymerase chain reaction; green icons are clean processes; red icons are dirty processes]

Figure 12. Steps in deoxyribonucleic acid extraction and quantitative polymerase chain reaction process designated as "clean" or "dirty."

after minor aerosolization events. For this reason, the most critical spaces to keep physically isolated from those used to manipulate clean reagents are those where amplicons are generated, analyzed (for example, by electrophoresis), or purified (Persing, 1991; Hopkins, 2008).

Careful use of equipment, reagents, and consumables can also help reduce the possibility of laboratory contamination. By categorizing the various steps of the qPCR process as either "clean" or "dirty," it is possible to eliminate unnecessary movement of instruments and materials between so-designated spaces. Pipettes, tips, plastics, and bench-top instruments such as centrifuges or vortexers should be reserved within the spaces provided for "clean" and "dirty" activities without the need for being transported from one part of the laboratory to another; for example, equipment used to make qPCR master mix should always remain in that "clean" space, whereas other instruments for adding template nucleic acid to the qPCR reaction plate are kept in a different space (Conraths and Schares, 2006; Hopkins, 2008). Positive controls, primers, probes, and other qPCR reagents should be aliquoted for single use whenever possible to avoid potentially introducing contaminants through repeated pipetting (Persing, 1991; Hopkins, 2008). Using a BSC can help prevent dispersing aerosolized microorganisms or contaminants, and changing laboratory coats and shoe coverings when entering a "clean" space from a "dirty" space can help reduce the chance of personnel inadvertently contaminating reactions via their clothing (Hopkins, 2008).

Finally, unidirectional workflow plays an important role in minimizing potential contamination in the molecular diagnostic laboratory (fig. 12). Limiting or restricting the physical movement of personnel from "dirty" to "clean" spaces is one approach laboratories may take to prevent the spread of contaminants. This approach may mean assigning individuals to certain steps in the process for a period (in other words, each day one designated employee makes all the qPCR master mixes) and doing thorough decontamination of all areas at the end of that period before a new person enters the space. When this approach is not feasible because of space or personnel restrictions, laboratories can ensure that technical staff are thoroughly trained to minimize contamination within and between workspaces. Such strategies include pipetting techniques that minimize splashes or aerosolization; effective use of BSC; appropriate methods for handling, transporting, and opening sample tubes; correct use and disposal of PPE including gloves, gowns, and masks; and doing work in a manner that minimizes movement from areas that are "dirty" to those that are "clean" (fig. 10; Hopkins, 2008).

Despite implementing prevention practices, occasional laboratory contamination is inevitable given the ability of molecular techniques to detect even minute amounts of target material. To address this eventuality, laboratories can institute practices to alert personnel to the potential presence of contamination in samples and to reduce the likelihood of reporting results incorrectly; for example, regular environmental swabbing of laboratory surfaces and equipment can provide advanced warning for the presence of contamination, as well as illuminate potential patterns of contamination that can be addressed through workflow amendments, additional training, or other remediation actions. In addition to the use of negative controls throughout the sample preparation and testing process, a "premises control" (an open tube of master mix placed in work areas and analyzed on a regular basis) can also serve as an early warning system for laboratory contamination problems via aerosols (Conraths and Schares, 2006).

Facilities within the WNS Diagnostic Laboratory Network are expected to make every reasonable effort to minimize the chance of laboratory contamination and to follow practices that will alert laboratory personnel to its presence to reduce risk of reporting a false-positive result. Recommended practices include, but are not limited to, those detailed in this chapter. Additionally, each laboratory should document, within their QMS, the steps they are taking to prevent, detect, and resolve contamination issues. Not only does this documentation ensure confidence in a given laboratory's results, but it also provides a starting point for troubleshooting should discrepancies or questions arise.

Pseudogymnoascus destructans Molecular Detection Methods (Deoxyribonucleic Acid Extraction and Quantitative Polymerase Chain Reaction)

Commercially available diagnostic tests that are not approved by the Food and Drug Administration (for human medicine) or the USDA (for veterinary medicine) are known as "laboratory-developed tests" (Burd, 2010). For human diagnostics, these tests are regulated by the Clinical Laboratory Improvement Amendments of 1988 (42 CFR 493.3), which require demonstration and documentation of benchmark performance characteristics (U.S. Department of Health and Human Services, 1992; Burd, 2010). Commercially licensed animal diagnostics are not subject to Clinical Laboratory Improvement Amendments oversight but are regulated by the USDA Veterinary Services Center for Veterinary Biologics, which defines required performance assessment criteria (APHIS, 2015). Diagnostic assays for primary use in wild animals are subject to much less oversight. The WOAH does provide guidelines for wildlife diagnostic assay validation, but rather than being mandated, they are simply encouraged as best practices (WOAH, 2018d). This lack of regulatory oversight for wildlife diagnostics can lead to inconsistencies in testing and reporting by different laboratories or institutions.

Laboratories have different reasons for changing elements of a molecular assay including established institutional relationships with vendors of certain products, differences in equipment and compatible commercial reagents, the need to conform to in-house operating standards, or limitations related to personnel time or expertise. According to the WOAH, alterations such as changes in equipment, reaction conditions, or chemistry are considered minor and acceptable provided a comparison test can demonstrate equivalent performance between previously established and modified methodologies (Bustin, 2010; WOAH, 2018c, b). When assay performance characteristics are reported in the scientific literature, this information can be used to document expected testing outcomes; however, variations to assays implemented by laboratories may not be published or otherwise communicated, leaving gaps in our understanding of how assays perform under the parameters and conditions used by different institutions. Within the context of a laboratory network, periodic internal or external assessments can help shed light on how these different testing parameters affect diagnostic performance and inform the establishment of laboratory standards for interpreting and reporting results to further harmonize testing outcomes.

Several qPCR assays have been described for detection of *Pd* (Chaturvedi and others, 2011; Muller and others, 2013; Shuey and others, 2014), but all laboratories testing for this fungus as part of the WNS Diagnostic Network have adopted the assay published by Muller and others (2013) (hereafter referred to as the "Muller assay"). The Muller assay is a TaqMan (probe-based) qPCR assay that targets the intergenic spacer region of the ribosomal ribonucleic acid (typically called RNA) gene complex (Espy and others, 2006; Muller and others, 2013). Shortly after the initial publication of the Muller assay, subsequent research demonstrated increased quantification accuracy by setting the qPCR threshold baseline to 4 percent of the maximum fluorescence rather than 10 percent as previously described (Muller and others, 2013; Verant and others, 2016). Adjusting the baseline fluorescence is currently the only published deviation to the original Muller assay qPCR protocol; however, through the years, the *Pd* assay has undergone many unpublished modifications, both in application and execution. As part of the harmonization process, *Pd* qPCR methodologies and parameters were collected from members of the WNS Diagnostic Laboratory Network (table 5). This informal survey revealed variations in how the assay was implemented and interpreted. Out of eight responding laboratories using the Muller assay, only two reported using the original published parameters. Two laboratories used different qPCR platforms (thermocycler machines), four used different commercial master mixes, and three noted changes to the published cycling parameters (table 5).

Table 5. Published and unpublished variations to the deoxyribonucleic acid extraction and quantitative polymerase chain reaction *Pseudogymnoascus destructans* assay parameters in use within the White-Nose Syndrome Diagnostic Laboratory Network.

[All quantitative polymerase chain reaction modifications listed were evaluated through interlaboratory testing and performed comparably in nucleic acid detection. Laboratories using parameters not included here should do in-house testing to ensure the modifications do not affect assay performance. qPCR, quantitative polymerase chain reaction; °C, degrees Celsius; ng, nanogram; ag, attogram; fg/µL, femtograms per microliter]

Parameter	Muller assay protocol parameters and published deviations	Protocol variants currently (2023) in use within the White-Nose Syndrome Diagnostic Laboratory Network
Extraction method(s)	● Tissue Samples and Fungal Culture: Gentra Puregene Genomic DNA Purification Kit (QIAGEN Inc.) and OmniPrep for Fungi (G-Biosciences)[1] ● Sediment: Powerlyzer PowerSoil DNA Isolation Kit (QIAGEN Inc. [formerly MO BIO Laboratories Inc.]), *plus* 30-minute heated-shaker incubation in lyticase[2,3] ● Environmental Swabs: MagMax Total Nucleic Acid Isolation Kit (Life Technologies Inc.), *plus* 30-minute heated-shaker incubation in sorbitol buffer containing lyticase and beta-mercaptoethanol[2,3] ● Skin and Environmental Swabs: DNeasy Blood & Tissue Kit – supplementary yeast protocol [30-minute incubation in sorbitol buffer containing lyticase and beta-mercaptoethanol] (QIAGEN Inc.)[4]	● MagMax Pathogen RNA/DNA (Life Technologies Inc.). ● MagMax Pathogen RNA/DNA (Life Technologies Inc.) *plus* initial 30-minute incubation in sorbitol buffer containing Zymolase and beta-mercaptoethanol followed by bead beating. ● DNeasy Blood & Tissue Kit, normal protocol and supplementary yeast protocol (QIAGEN Inc.). ● QIAGEN QIAamp DNA Mini Kit (QIAGEN Inc.). ● Powerlyzer PowerSoil DNA Isolation Kit (QIAGEN Inc. [formerly MO BIO Laboratories Inc.]). ● PrepMan Ultra Sample Preparation Reagent (Applied Biosystems) with mechanical disruption using zirconium/silica beads (BioSpec Products) with and without heat. ● MagNA DNA/Viral SV 2.0 kit (Roche) with TriReagent.
qPCR reagents	● QuantiFast Probe PCR + ROX Vial Kit (QIAGEN Inc.)[1] ● QuantiFast Probe PCR + ROX Vial Kit (QIAGEN Inc.) plus bovine serum albumin (Sigma-Aldrich)[2]	● Applied Biosystems Path-ID qPCR Master Mix (Thermofisher, ABI). ● Applied Biosystems AgPath-ID One-Step RT-PCR kit (Thermofisher, ABI). ● QuantiFast Probe PCR + ROX Vial Kit (QIAGEN Inc.).[*] ● QuantiFast Prope PCR + ROX Vial Kit (QIAGEN Inc.) plus bovine serum albumin (Sigma-Aldrich).[†] ● LightCycler 480 Probes Master.
Thermocycler platform	● ABI 7500 Fast Real-Time PCR System (Life Technologies Inc.)[1,2,4]	● Applied Biosystems 7500 Fast Real-Time PCR System. ● Applied Biosystems StepOnePlus Real-Time PCR System. ● Roche LightCycler 480.
Cycling parameters	● Polymerase activation at 95 °C for 3 minutes followed by 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds[1,2]	● Original Muller assay cycling parameters ● Polymerase activation at 95 °C for 10 minutes followed by published cycling times/temperatures. ● Initial denaturation at 50 °C for 2 minutes and polymerase activation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. ● Original polymerase activation followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds.
Threshold baseline	● 10 percent maximum fluorescence[1] ● 4 percent maximum fluorescence[2]	● Set automatically by qPCR machine. ● 10 percent maximum fluorescence. ● 4 percent maximum fluorescence.

Table 5. Published and unpublished variations to the deoxyribonucleic acid extraction and quantitative polymerase chain reaction *Pseudogymnoascus destructans* assay parameters in use within the White-Nose Syndrome Diagnostic Laboratory Network.—Continued

[All quantitative polymerase chain reaction modifications listed were evaluated through interlaboratory testing and performed comparably in nucleic acid detection. Laboratories using parameters not included here should do in-house testing to ensure the modifications do not affect assay performance. qPCR, quantitative polymerase chain reaction; °C, degrees Celsius; ng, nanogram; ag, attogram; fg/µL, femtograms per microliter]

Parameter	Muller assay protocol parameters and published deviations	Protocol variants currently (2023) in use within the White-Nose Syndrome Diagnostic Laboratory Network
Controls and Standard Curves	• Standard curve consisting of 1:10 serial dilutions from 3.3 ng to 3.3 ag genomic DNA (gDNA), along with positive (gDNA) and negative (no template) amplification controls[1] • Standard curve consisting of 1:10 serial dilutions from 3.3 ng to 3.3 fg gDNA along with positive (gDNA) and negative (no template) amplification controls and negative (no conidia) extraction control[2]	• 1:10 8-point standard curve (gDNA) at beginning of sample season for calibration, and 3-point curve consisting of 1:10 serial dilutions from 20,000 to 200 fg/µL per plate. • 1:10 8-point standard curves (gDNA) at beginning of sample season for calibration, and single positive amplification control (gDNA) per plate. • 6-point standard curve (gDNA) per plate. • 1:10 6-point standard curve (gBlocks gene fragment, Integrated DNA Technologies) consisting of 250 to 0.0025 fg/µL in triplicate per plate. • Negative extraction controls (no conidia), between 1 and 8 per plate (varies by laboratory). • Negative amplification controls (no template), between 1 and 13 per plate (varies by laboratory). • Commercial Internal Positive Control (VetMAX Xeno Internal Positive Control RNA, Applied Biosystems). • Positive extraction controls (matrix spiked with conidia). • Positive amplification controls (gDNA or gBlocks gene fragment, Integrated DNA technology).

[1]Muller and others (2013).

[2]Verant and others (2016).

[3]Authors tested multiple modifications to several commercial kits. Protocols listed in this table outperformed other variations in terms of nucleic acid recovery, thus lower performing variations have been omitted.

[4]Originally published by Shuey and others (2014) and subsequently used by multiple studies in conjunction with the Muller assay (Muller and others, 2013).

Interlaboratory testing was done in 2019 and 2021 to evaluate the effect of these different in-house modifications on assay performance and consistency of reported results. In 2019, limits of detection (LOD) testing took place at five North American laboratories (two in Canada, and three in the United States) with data collation and analysis done by the USGS National Wildlife Health Center (Madison, Wisconsin). All data collected for this study are available in a USGS data release (Alger and others, 2023). This was the first formal evaluation of how different *Pd* qPCR testing alterations may contribute to variability in results among laboratories. The process enabled the DxWG to take a consensus-based, data-driven approach to making interpretation standards and reporting language more consistent across the WNS Diagnostic Laboratory Network. In 2021, 8 North American laboratories (2 in Canada and 6 in the United States) participated in an expanded round of testing requiring DNA extraction and qPCR on 28 blinded samples. The following sections provide some generalized conclusions within the context of understanding how the DxWG established baseline metrics for the performance of the *Pd* qPCR assay among WNS Diagnostic Network Laboratories, as well as for the purposes of centralizing this information within this handbook.

Extraction Methods

The method used to extract DNA from a diagnostic sample can directly affect overall diagnostic performance. Multiple studies have demonstrated statistically significant differences in DNA recovery and as much as a 31-percent range in an assay's ability to successfully identify positive samples based upon the extraction procedure used (Fredricks and others, 2005; Bletz and others, 2015; Norman and Dinauer, 2016). One advantage of qPCR is the ability to use the resulting Ct values to quantify the amount of starting target in a reaction (Life Technologies, 2015). This quantity is, in turn, used to

infer the amount of target nucleic acid present in the original sample. When extraction method efficiency is not considered, this inference may be skewed (Fredricks and others, 2005; Kralik and Ricchi, 2017). Additionally, in samples with low amounts of target, poor extraction efficiency can cause false negatives by not recovering an adequate amount of DNA for amplification (Maaroufi and others, 2004; Johnson and others, 2013). As mentioned previously, extraction procedures for fungal samples (such as *Pd*) have additional challenges. Fungi have cell walls that resist lysis, often requiring multiple methods of disruption (such as chemical, mechanical, and (or) enzymatic) for DNA recovery (Maaroufi and others, 2004; Fredricks and others, 2005; Karakousis and others, 2006). Sample type should also be considered when developing optimal extraction methods. Some matrices, such as soil and feces, contain substances that inhibit qPCR amplification and must be removed during the extraction process (Wilson, 1997). In addition, different sample types may favor the presence or persistence of certain fungal life stages (in other words, hyphae versus conidia) with variable cellular wall composition and, therefore, different optimal lysis methods (Fredricks and others, 2005; Verant and others, 2016, Garcia-Rubio and others, 2020; Urbina and others, 2020).

Although standardizing *Pd* extraction methods across the WNS Diagnostic Network would be an optimal solution, most laboratories use commercially available kits that may differ in price and processing time. Depending upon available laboratory infrastructure and resources, use of some commercial kits may not be feasible, particularly when high volumes of samples are anticipated. Verant and others (2016) previously tested four different commercial nucleic acid extraction kits for the detection of *Pd* in swabs and environmental samples. Each kit was evaluated for DNA recovery using the manufacturer's original protocol and a set of amended protocols to improve cell lysis (Verant and others, 2016). The extraction kits that produced the highest quality and quantity of DNA were those that were amended to include multiple disruption methods, demonstrating the importance of method evaluation to determine whether modifications to manufacturer protocols are warranted (Verant and others, 2016). In the future, interlaboratory proficiency testing can be designed in a way that will allow for more direct comparison of extraction methods between members of the WNS Diagnostic Laboratory Network. In the meantime, differing extraction methods may be a source of interlaboratory testing variability and should (minimally) be evaluated by each laboratory during the assay optimization process (Norman and Dinauer, 2016).

Pseudogymnoascus destructans Quantitative Polymerase Chain Reaction Assay

When assessing the performance of an assay, several metrics are used, some of which are analytical and refer to the performance of the assay under laboratory conditions and others of which are diagnostic and refer to the performance of the assay in the population of interest (Flatland and others, 2014). Sensitivity and specificity are terms that can be applied to the analytical and diagnostic characteristics of an assay; therefore, clarity about the system of measurement being described is important (Saah and Hoover, 1997). In the following section, background on common analytical and diagnostic parameters and an overview of their application to the evaluation of *Pd* qPCR performance are provided. A discussion follows on how appropriate interpretation of these metrics may aid in the communication of results through a better understanding of test uncertainty and assay limitations.

Analytical Sensitivity (Limit of Detection)

Analytical sensitivity is defined as the smallest amount of analyte detectable at a given threshold—typically in 95 percent of replicates, although other levels may be used with justification (Burd, 2010; Persing and others, 2016; WOAH, 2018e). Analytical sensitivity and LOD are typically referred to synonymously. Laboratories can determine the LOD for qPCR assays in one of two ways. The first, known as a "benchtop" method, involves doing qPCR analyses on repeated serial dilutions. The LOD is the lowest dilution at which 95 percent (or other prestated threshold) of the replicates are positive, meaning target DNA was detected (Burd, 2010; Persing and others, 2016). For this method, the dilution factor will affect the precision of the LOD estimate (in other words, the smaller the dilution factor, the closer to the true value; Caraguel and others, 2011), and the number of replicates will improve the accuracy of the LOD estimate (in other words, the greater the number replicates, the less effect false negatives will have on the result; Persing and others, 2016). Recommendations for number of replicates varies in the range of 8–20 per dilution (Burd, 2010; Persing and others, 2016).

The second method determines the exact LOD through modeling the probability of detection (Burns and Valdivia, 2008; Forootan and others, 2017). This method also uses serial dilutions, but the range of dilutions would need to include concentrations of analyte that are beyond the anticipated LOD and, therefore, expected to be undetectable by the assay. Although this method is more accurate, results are also more sensitive to the number of replicates per dilution (Caraguel and others, 2011; Forootan and others, 2017; Klymus and others, 2020). Because of the stochasticity associated with the Poisson sampling distribution, the nature of qPCR reactions, and the probability of including at least one copy in the reaction, the lowest theoretical LOD is three copies (Bustin and others, 2009).

When the Muller assay was first published (Muller and others, 2013) and subsequently amended (Verant and others, 2016), the smallest amount of nucleic acid material that consistently amplified in 3 out of 3 replicates was determined to be 3.3 femtograms (fg) of purified *Pd* genomic DNA. However, quantification of genomic DNA is not consistent between isolates of *Pd* because of the variable number of gene

copies within the intergenic spacer region, making it difficult to translate this result into more a more standardizable unit, such as copy number (Muller and others, 2013).

Other studies have demonstrated detecting smaller amounts of *Pd* DNA in samples (Urbina and others, 2020), and results of the 2019 interlaboratory testing demonstrated that when using synthetic sequences (such as gBlocks) the actual limit of detection across laboratories is closer to 3 copies per microliter (box 4; table 6). Note that these extreme low levels were achieved under ideal conditions, using DNA fragments designed specifically for amplification and prepared in a matrix known to be free of inhibitors. Because these conditions differ from those found when processing field samples, these limits may be too low to be useful from a diagnostic standpoint. They do, however, establish baseline performance measures across multiple laboratories, platforms, and methods that demonstrate how diagnostic results from different institutions may be meaningfully compared; for example, the variation around the mean (also known as the coefficient of variation; CV) for Ct values across laboratories were more variable than quantified copy numbers (table 7). This implies that when multiple laboratories test the same sample, the resulting Ct values should first be converted to a metric such as sequence copies or femtograms to ensure interlaboratory comparisons are accurate. The interlaboratory testing also indicated that analytical sensitivity did not differ substantially across laboratories, providing the sought-after assurance that individual laboratory modifications to the Muller assay do not seem to have adversely affected its analytical performance and that all laboratories are capable of successfully amplifying very small amounts of target DNA (table 6).

Limit of Quantification

Limit of quantification (LOQ) is an analytical performance measurement that refers to the lowest amount of analyte that can be accurately measured (Burd, 2010). This value may be equal to, but never lower than, the LOD and is typically delineated by the point where the CV for replicates exceeds a predetermined amount (Kralik and Ricchi, 2017). As noted earlier, the method for calculating CV differs depending on whether the unit of measurement is linear (weight or number of target DNA copies produced) or logarithmic (Ct), and using the wrong formula will result in an inaccurate estimation of variability (Kubista, 2014; Canchola and others, 2017; Forootan and others, 2017; table 7). There is no universal guidance on what the CV among replicates for a diagnostic qPCR assay should be, but studies have cited values between 25 and 35 percent (Kubista, 2014; Forootan and others, 2017; Kralik and Ricchi, 2017). The LOQ can also be estimated by examining the assay's standard curve to determine the lowest dilution (in other words, the highest Ct value) at which the replicates lose linearity (Burd, 2010). Knowing the LOQ of an assay is critical when reporting high Ct (low analyte) values. Because results beyond the LOQ (CV greater or equal to [≥] 35 percent) are highly variable, quantification for high Ct results should not be reported without adequate communication to describe the uncertainty of the calculated values (Burd, 2010; Klymus and others, 2020).

For the *Pd* qPCR assay, LOQs were formally evaluated for the first time in 2019 through interlaboratory testing (box 4). Results indicated LOQ values among laboratories ranged from about 2–11 copies per microliter (table 6), which is consistent with other findings on the effects of assay parameter variation (Klymus and others, 2020). Further work could be completed to identify why LOQ is variable among laboratories; however, the number of assay parameter variations currently in use could make this difficult. In addition, most laboratories within the WNS Diagnostic Network report Ct values with or without qualitative categorization and do not convert their Ct values into quantified estimates for reporting. Because of this, the DxWG has not established common thresholds for translating quantitative results into qualitative categories, as was done with Ct values. Although this step may be necessary in the future if more laboratories are interested in reporting DNA quantities, at this point it is more important for individual laboratories to simply be aware of their in-house LOQ and either report values that fall below the LOQ qualitatively or clearly communicate the uncertainty associated with quantified estimates in that range. Additional information is provided in the "*Pseudogymnoascus destructans* Quantitative Polymerase Chain Reaction Result Interpretation" subsection of the "*Pseudogymnoascus destructans* Quantitative Polymerase Chain Reaction Assay" section.

Analytical Specificity

Analytical specificity is the ability of an assay to correctly identify the target analyte without cross-reacting with other substances in a way that interferes with accurate detection or quantification (Burd, 2010; Wolk and Marlowe, 2016; WOAH, 2018e). For molecular assays, analytical specificity refers to the ability of the test to differentiate between the target sequences and the sequences that represent closely related organisms by failing to amplify the latter (Burd, 2010). Analytical specificity should be evaluated early in assay development to ensure that the test includes all strains and lineages associated with the organism of interest while also excluding any similar, nontarget DNA that should be excluded for diagnostic purposes (WOAH, 2018e). Careful design of primers and probes, as well as thorough phylogenetic analysis during assay development, helps to ensure this performance metric is adequately met.

Table 6. Effective limits of detection results from round 1 of interlaboratory testing among five White-Nose Syndrome Diagnostic Laboratory Network members.

[Data are summarized from Alger and others (2023). LOD, limit of detection; CI, confidence interval; LOQ, limit of quantification]

Laboratory	gBlocks *Pseudogymnoascus destructans* sequence copies per microliter				
	LOD	Standard error	Lower 95-percent CI	Upper 95-percent CI	LOQ
Laboratory A	2.85	0.89	1.08	4.61	4
Laboratory B	1.50	0.56	0.37	2.62	2
Laboratory C	2.77	0.49	1.8	3.74	11
Laboratory D	2.36	0.48	1.40	3.32	9
Laboratory E	2.87	0.60	1.67	4.06	10
All laboratories	3.08	0.48	2.14	4.02	6

Table 7. Average cycle threshold value and copy estimate from interlaboratory limit of detection testing using data from all laboratories combined.

[Data are summarized from Alger and others (2023). Copy estimates were calculated by standard curve for each laboratory. The coefficient of variation (CV) for the copy estimates (linear) was calculated by dividing the standard deviation (σ) estimate of each dilution by the mean (μ) and multiplying by 100 to convert to a percentage: $CV = (\sigma/\mu) \times 100$. The CV for the cycle threshold (Ct) values (logarithmic) was calculated using the following formula described by Forootan and others (2017): $CV_{ln} = \sqrt{(1+E)^{(SD(Ct))^2 * ln(1+E)} - 1}$, where E is the qPCR efficiency and $SD(Ct)$ is the standard deviation of the replicate Ct values. Additional information on calculating and interpreting CV is found in the “Limits of Quantification” section. Min, minimum; Max, maximum; µL, microliter; *Pd*, *Pseudogymnoascus destructans*]

Copies per µL	Average (µ)	Standard deviation (σ)	*CV*, in percent	Min	Max
Raw Ct values					
34.12	32.00	1.47	129	29.03	34.35
17.06	33.10	1.49	133	30.09	35.94
8.53	33.80	2.01	230	30.12	37.80
4.26	34.85	1.90	205	31.54	37.71
2.13	36.00	1.98	224	32.31	40.00
1.07	36.38	1.81	186	33.08	40.36
Pd copy estimates					
34.12	35.55	8.11	23	17.97	58.31
17.06	17.32	4.45	26	8.00	27.39
8.53	8.56	2.87	34	2.12	17.31
4.26	4.30	1.57	37	1.13	9.45
2.13	2.03	1.18	58	0.28	6.67
1.07	1.32	0.66	50	0.29	3.58

Box 4. Limits of Detection and Quantification Testing Protocol

This protocol for testing the analytical sensitivity and limits of detection for the *Pseudogymnoascus destructans* quantitative polymerase chain reaction assay was developed by the White-Nose Syndrome Diagnostic Working Group and performed for the first time by members of the White-Nose Syndrome Diagnostic Laboratory Network in 2019. Synthetic gene fragments (gBlocks) were obtained through Integrated DNA Technology (https://www.idtdna.com/pages) using the following sequence: /5 TCT AGT CAG CCT CTC TGG TGG CCT CTG CCT CTC CGC CAT TAG TGC CGG TGT AGC TGG CGT TAC AGC TTG CTC GGG CTG CCT CTC TAG CTG GTT TTG CCG TGG TAG CTC ACC TAC CTA GCG AGC CGG TGG TGG CTG CTT TGC CG /3. Lyophilized fragments were sent to participating laboratories in quantities of 250 nanograms (ng) with instructions for resuspension, using 1× Tris-ethylenediaminetetraacetic acid (known as TE) buffer, to a concentration of 10 nanograms per microliter (ng/µL; tables B4.1, B4.2). Expected copy numbers per microliter were calculated as follows, based on the gBlocks molecular weight of 88,251.8 grams per mol (g/mol):

Copies per femtogram = ([6.022×10^{23} copies per mol)/(88,251.8 grams per mol)/(10^{15} femtograms per gram])

Table B4.1. Dilution series for interlaboratory testing to determine limits of detection.

[Serial dilutions 3, 2, 1, 0, −1, and −4, also shown in blue, are used for standard curve construction. Serial dilutions A–F, also shown in green, are used for limit of detection (LOD) estimation. Initial working stock of 1 nanogram per microliter (ng/µL) based on 1:10 dilution of 10 ng/µL gBlocks rehydration concentration was used per manufacturer instructions; TE, Tris-ethylenediaminetetraacetic acid]

Dilution label	Femtogram per microliter	Copies per microliter	Microliter from previous dilution	Microliter from 1× TE buffer or nuclease-free water
5	100,000	682,380,000	5 (1ng/µL working stock)	45
4	10,000	68,238,000	10	90
3	1,000	6,823,800	10	90
2	100	682,380	10	90
1	10	68,238	10	90
0	1	6,824	10	90
−1	0.1	682.38	10	90
−2	0.01	68.24	10[a]	90[a]
−3	0.001	6.82	10	90
−4	0.0001	0.68	10	90
A	0.005	34.12	70 of dilution −2[a]	70[a]
B	0.0025	17.06	70	70
C	0.00125	8.53	70	70
D	0.000625	4.26	70	70
E	0.000313	2.13	70	70
F	0.000156	1.06	70	70

[a]For series used in LOD calculation, dilution factor changes from 1:10 to 1:2, using the 0.01 femtogram per microliter (label −2) dilution as the initial base.

Box 4. Limits of Detection and Quantification Testing Protocol—Continued

Table B4.2. Example plate layout with dilution tube labels shown for each well.

[Wells for serial dilutions A–F, also shown in green, are used for standard curve construction. Wells for serial dilutions G and H, also shown in blue (except for cells with “NTC”), are used for limit of detection estimation. NTC, no template control (negative control)]

Dilution label	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	A	A	A	A	A	A	A	A	A	A
B	B	B	B	B	B	B	B	B	B	B	B	B
C	C	C	C	C	C	C	C	C	C	C	C	C
D	D	D	D	D	D	D	D	D	D	D	D	D
E	E	E	E	E	E	E	E	E	E	E	E	E
F	F	F	F	F	F	F	F	F	F	F	F	F
G	3	3	2	2	1	1	0	0	−1	−1	−4	−4
H	−1	−1	−4	−4	−4	−4	−4	−4	−4	−4	NTC	NTC

Numerous studies have examined the phylogenetic relationships between *Pd* and other closely related fungal strains (Gargas and others, 2009; Lindner and others, 2011; Lorch and others, 2013a). The first molecular diagnostic test for *Pd* was a nonquantitative PCR test that was able to detect *Pd* on bat wing skin with high diagnostic specificity (Lorch and others, 2010); however, when soil samples were examined, this assay also amplified nontarget DNA from other closely related *Geomyces* and *Pseudogymnoascus* species, and Sanger sequencing of PCR amplicons was required to differentiate these near-neighbor fungal species from *Pd* (Lindner and others, 2011; Minnis and Lindner, 2013). This limitation was addressed during development of the current, widely used Muller assay by targeting a different part of the ribosomal RNA gene region with greater variability between species (Jackson and others, 1999; Muller and others, 2013). The result was a more analytically specific qPCR diagnostic test that was much less likely to cross-react with DNA from nontarget organisms (Muller and others, 2013).

As samples are increasingly collected from new geographic areas without documented history of the pathogen’s presence, sequencing may play a greater role in verifying positive results. Several studies have characterized environmental fungi from bat hibernacula in eastern North America, but surveys of hibernacula in the western United States have to date (2022) primarily focused on bat host populations rather than on microbial communities (Lindner and others, 2011; Lorch and others, 2013a, b; Minnis and Lindner, 2013; Weller and others, 2018). To date, the Muller assay has continued to demonstrate high specificity for *Pd* from samples representing a wide geographic range, indicating that false-positive results because of cross-reactivity with fungi other than *Pd* are unlikely (Muller and others, 2013; Barlow and others, 2015; Bernard and others, 2015; Hoyt and others, 2016); however, because of the uncharacterized microbiome of newly invaded areas, cross-reactivity cannot be completely ruled out. It may therefore be prudent for laboratories to have a plan for confirming *Pd* (such as sequence analysis of PCR amplicons) when positive results are discovered in new geographic areas or on new species (Muller and others, 2013; Lorch and others, 2016).

Repeatability and Reproducibility

Repeatability and reproducibility are measures of precision and accuracy between assay runs and are typically measured in CV of copy number or concentration estimates (Bustin and others, 2009). Repeatability is a measure of precision between results in the same run or between runs for a single laboratory (Viljoen and others, 2005; WOAH, 2018c). Within run (intra-assay) repeatability is measured by including multiple standards of equal, known concentration on the same plate. Between run (interassay) repeatability is measured by including a positive control of known concentration on multiple plates (Kralik and Ricchi, 2017). Repeatability is measured by calculating the CV of the results for each standard (Taylor and others, 2019). A benchmark of no more than 15 percent CV has been suggested for repeatability estimates (Burd, 2010); however, given that CV will increase as target analyte amounts decrease because of inherent stochasticity in subsampling (replicates) associated with the Poisson distribution, a less rigorous benchmark of 25–35 percent CV may be more acceptable for standards with smaller amounts of starting target (Wolk and Marlowe, 2016; Taylor and others,

2019). Because of this variation in repeatability across a range of detectable concentrations, laboratories are encouraged to estimate the precision of their assay at low, medium, and high levels of analyte.

Reproducibility is the measure of precision between results obtained by different laboratories using the same test material (Bustin and others, 2009; Kralik and Ricchi, 2017). Similar to repeatability, reproducibility is evaluated by calculating the CV of copy number or concentration estimates between laboratory results for the same standard concentration. Unlike repeatability, however, the degree of variability between laboratories for samples with very low concentrations of target nucleic acid (less than 100 copies) is expected to be much higher. This is because the variation in subsampling error between different laboratories because of equipment calibration or user method translates to a higher effect on the results of these low-level samples (Taylor and others, 2019); therefore, there is precedent in some industries, such as the molecular detection of genetically modified organisms in food products, to accept CV values as high as 50 percent for samples with extremely low expected concentrations of target nucleic acid (European Network of GMO Laboratories, 2015).

Laboratories within the WNS Diagnostic Laboratory Network can monitor the repeatability of their in-house assay by including in each testing run a known standard consisting of fungal conidia in matrix that is similar to the diagnostic samples being tested. This standard should be processed through the same procedure as the diagnostic samples from extraction to amplification (Kralik and Ricchi, 2017; WOAH, 2018c). This record of extraction control performance can be part of the laboratory's QMS (see the "Controls" subsection of "Best Practices—Laboratory") to better communicate the degree of uncertainty related to results, and to indicate when quantification is no longer accurate. When the extraction control value begins to fluctuate beyond the laboratory's predetermined CV threshold, laboratories should investigate the loss of repeatability and correct problems that may be contributing to diminished assay performance.

Reproducibility for the *Pd* qPCR assay was evaluated via interlaboratory testing in 2019, along with LOD and LOQ (box 4; table 8). To better understand potential discordant results between laboratories, two metrics were used to assess reproducibility for each standard concentration across laboratories: precision (CV) and detection rate (number of replicate detections/replicates per standard). Results indicated consistent reproducibility precision (CV less than or equal to 35 percent) and consistency (high detection rate) among laboratories for all samples except those at the lowest concentrations of target DNA (table 8). The increased variability in precision for low-analyte samples (about 4 copies or less per microliter; table 8) underscores challenges associated with obtaining independent verification of high Ct amplification results. Samples with small amounts of target nucleic acid produce results that are inherently less consistent across replicates due to the Poisson distribution, leading to confusion when such results are interpreted as positive or inconclusive but cannot be subsequently reproduced. For this reason, the most recent version of the WNS case definition recommends that samples yielding high Ct values (greater than or equal to 37) not be sent to other laboratories for confirmatory testing (WNS DxWG, 2021). Assay reproducibility will continue to be assessed within the network via proficiency testing, and new laboratories joining the WNS Diagnostic Laboratory Network should demonstrate a comparable degree of reproducibility to other network laboratories. Laboratories wishing to participate in the WNS Diagnostic Laboratory Network should also consider performing in-house LOD testing according to the DxWG's established protocol (box 4) to demonstrate that any modifications they have made to the assay do not interfere with its performance and that they can achieve analytical performance consistent with the other network laboratories.

Table 8. Average copy estimates by dilution for each laboratory and all laboratories combined from 2019 interlaboratory testing for *Pseudogymnoascus destructans* quantitative polymerase chain reaction Muller and others (2013) assay.

[Data are summarized from Alger and others (2023). Results are highly precise (coefficient of variation around the mean [CV] less than or equal to 35 percent) and detection rates (noted in parenthesis as number of replicate detections/number of replicates per standard) are consistent among laboratories, even at low concentrations, indicating good reproducibility for the assay within the White-Nose Syndrome Diagnostic Laboratory Network. μ, mean; σ, standard deviation]

Copies	Lab A	Lab B	Lab C	Lab D	Lab E	All μ	All σ	CV, in percent
34.12	29.06 (12/12)	35.88 (12/12)	33.83 (12/12)	43.19 (12/12)	30.37 (12/12)	35.55 (60/60)	8.11	23
17.06	13.74 (12/12)	17.26 (12/12)	17.61 (12/12)	20.21 (12/12)	13.70 (12/12)	17.32 (60/60)	4.45	26
8.53	8.95 (24/24)	8.35 (12/12)	6.98 (12/12)	7.64 (12/12)	8.24 (12/12)	8.56 (72/72)	2.87	34
4.27	3.97 (23/23)[1]	4.25 (12/12)	3.91 (11/12)	4.68 (12/12)	3.96 (12/12)	4.30 (70/71)	1.57	37
2.13	1.84 (23/24)	2.12 (12/12)	2.65 (8/12)	1.58 (11/12)	2.03 (10/12)	2.03 (64/72)	1.17	58
1.07	1.14 (19/24)	1.42 (10/12)	1.31 (4/12)	1.56 (6/12)	1.91 (5/12)	1.32 (44/72)	0.66	50

[1]One replicate had a cycle threshold (Ct) that was greater than 10 percent from the median Ct value for that dilution. The result was considered an outlier and excluded from further analysis.

Diagnostic Metrics

Diagnostic sensitivity and specificity are measures of performance accuracy and refer to the ability of a test to correctly identify individuals in a population as either clinically positive or negative (Kralik and Ricchi, 2017; box 5). In brief, diagnostic sensitivity (DSe) is the ability of a test to correctly identify individuals in a population with the given disease (true positives), whereas diagnostic specificity (DSp) is the ability of a test to correctly identify individuals without the disease (true negatives; Saah and Hoover, 1997). Diagnostic sensitivity and specificity are traditionally used to evaluate the ability of a screening test to detect a clinical disease or condition by sampling populations of known disease status (positive or negative) alongside a "gold standard" test that is considered the definitive diagnostic (Burd, 2010; Friis and Sellers, 2014; Wolk and Marlowe, 2016). For most screening tests, there is some degree of overlap between those with the disease or condition and the normal healthy population for the analyte in question (Simon and Boring, 1990). Because of this, DSe and DSp are almost never perfect, raising the possibility that some individuals will test falsely positive whereas others will test falsely negative. As a result, although these metrics provide some useful information about test performance, they provide no information for making an individual diagnosis (Trevethan, 2017; box 5).

Positive and negative predictive values, on the other hand, are additional metrics used to assess the diagnostic performance of a screening test in light of population prevalence for the disease or condition in question and can therefore offer more meaningful insight into the interpretation of an individual result (Saah and Hoover, 1997; Akobeng, 2007; Friis and Sellers, 2014). Positive predictive value indicates the probability that a positive test result accurately reflects the presence of disease. Negative predictive value indicates the probability that a negative test result accurately reflects the absence of disease. Because positive predictive value and negative predictive value integrate population prevalence into their calculation, extremes in these values (very high prevalence or very low prevalence) can strongly affect the interpretation of results within a specific population being tested (fig. 13).

Understanding Diagnostic Performance Metrics and the Role of Uncertainty

Diagnostic performance metrics are commonly used in assay development and epidemiology, but their application to qPCR requires a full understanding of the assumptions inherent in these calculations as well as the intended purpose of the test being evaluated. As mentioned previously, these metrics were developed to evaluate the ability of a test to correctly screen individuals for a disease or condition in comparison to a gold standard diagnostic test, assuming that overlapping ranges of test values in healthy and nonhealthy populations give rise to false positives and negatives (Simon and Boring, 1990; Flatland and others, 2014; Trevethan, 2017). As such, these metrics are most useful when applied to tests that screen for physiologic indicators of the condition (for example, elevated glucose for diabetes or radiographic evidence of joint deterioration for rheumatoid arthritis) or tests to identify viable pathogen presence within the host (for example, bacterial culture for *Mycobacterium tuberculosis*; Simon and Boring, 1990; Limmathurotsakul and others, 2010; Flatland and others, 2014; Friis and Sellers, 2014).

In contrast, DSe and DSp are less meaningful when no assumption can be made that pathogen detection equates with clinical disease, as in the case of many molecular assays, including *Pd* qPCR (Saah and Hoover, 1997; Akobeng, 2007; Burd, 2010). Molecular assays are designed to detect the presence of target genetic material within a host but are unable to identify physiologic processes associated with disease (such as inflammatory markers, tissue damage, change in blood chemistry, and so on) and cannot determine microbial viability (Persing and others, 2016). Given that the true state by which diagnostic performance measures are evaluated is disease presence, the inability of an assay to identify this state violates the basic assumptions of these metrics (Simon and Boring, 1990; Akobeng, 2007). In addition, many molecular tests—including the *Pd* qPCR—are used to detect potentially pathogenic organisms in nonhost material such as environmental, food, or water samples (Wolffs and others, 2005; Lorch and others, 2013a, b). Recent increases in the acceptance and use of environmental DNA (eDNA) surveys for everything from rare or invasive species to contaminants and pathogens has resulted in further shifts away from performance metrics based on disease to an increased use of statistical occupancy modeling for evaluating an assay's ability to detect its target (MacKenzie and Royle, 2005; Bailey and others, 2014; Hunter and others, 2017; Davis and others, 2018; Mize and others, 2019; Sepulveda and others, 2019; Klymus and others, 2020).

The frequent use of molecular assays to detect (rather than diagnose) has led to the adoption of the terms accuracy and trueness as more appropriate assessments of diagnostic performance (Espy and others, 2006; Persing and others, 2016). Accuracy is a broadly defined metric that describes the degree of agreement between a test result and the true (or assumed true) value or state (Burd, 2010; Kralik and Ricchi, 2017). By this definition, DSe and DSp are measures of clinical accuracy and may be appropriate for evaluating molecular assays when a clear link between the presence or amount of pathogen and clinical disease can be established (Steurer and others, 2002; Linnet and others, 2012). For many molecular assays, however, accuracy is considered a measure of the test's ability to determine pathogen presence or absence (Kralik and Ricchi, 2017), in which case additional information may be required for appropriate clinical interpretation (Burd 2010). For quantitative assays such as qPCR, accuracy refers to the degree of agreement between the expected amount of analyte and the measured amount, and is more commonly referred to as trueness (Burd, 2010; Kralik and Ricchi, 2017).

Box 5. Understanding Diagnostic Sensitivity and Diagnostic Specificity

Diagnostic sensitivity (DSe) and specificity (DSp) are useful metrics for understanding how a diagnostic test performs in a population but can cause confusion when not interpreted appropriately. Unlike analytical sensitivity and specificity that evaluate the ability of a diagnostic test to directly measure the presence or absence of a particular agent, DSe and DSp are used to evaluate tests that measure indicators of disease, but not the disease directly. These indicators are typically physiologic or metabolic in nature and are therefore present within a population to varying degrees, with or without associated pathology. When graphed, the range of potential measurement values (test results) in a population typically forms two distributions—one for individuals that have the disease and one for individuals that do not (fig. B5.1). The area of overlap between the two distributions shows the range of results that may indicate nondiseased (true negative) or diseased (true positive) individuals. The DSe and DSp of the test therefore depend upon where the delineation between positive and negative results are drawn. In figure B5.1, the cut-off in, *A*, maximizes DSe by using the lowest value in the diseased population distribution to determine a positive result. This cut-off ensures that all true positives will be successfully identified (100 percent DSe) but will also result in a relatively large number of false positives (true negatives that are categorized as positive). The cut-off in, *B*, maximizes DSp by using the highest value in the nondiseased population distribution to determine a positive result. This cutoff ensures that all true negatives will be successfully identified (100 percent DSp) but will also result in a relatively large number of false negatives (true positives that are categorized as negative). Most diagnostic cut-off values are chosen to simultaneously maximize both DSe and DSp while minimizing miscategorized results. These tests, shown in *C* have a Dse and DSp that are both less than 100 percent and will identify most true positives and negatives but will also mistakenly categorize a few of each.

Diagnostic sensitivity and specificity are common performance metrics of diagnostic tests but are commonly misinterpreted or confused with positive or negative predictive values. A study from the British Medical Journal that tested a cohort of 263 physicians on their understanding of these concepts found that nearly 25 percent of participants were unable to correctly define diagnostic sensitivity, and greater than 75 percent substantially overestimated the true probability of disease when given test sensitivity and population prevalence (Steurer and others, 2002).

A common error related to diagnostic sensitivity and diagnostic specificity is assuming that a highly sensitive test means a positive result is certain to be a true positive or, conversely, assuming that a highly specific test means that a negative result is certain to be a true negative. A test with 100-percent sensitivity and 95-percent specificity will correctly identify every individual with the disease or condition (no false negatives) but will potentially identify some healthy individuals as positive as well. In this scenario, a positive result could be either true or false, whereas a negative result could only be true. High sensitivity tests are, therefore, useful for ruling out conditions. Conversely, a test with 95-percent sensitivity and 100-percent specificity will correctly identify every individual without the disease or condition (no false positives) but will potentially identifying some diseased individuals as negative. In this scenario, a negative result could be true or false, whereas a positive result could only be true. High specificity tests are, consequently, useful for ruling in conditions. Two useful mnemonics for remembering this are: SnNOUT (high sensitivity, negative, rule out) and SpPIN (high specificity, positive, rule in) (Akobeng, 2007; Trevethan, 2017)

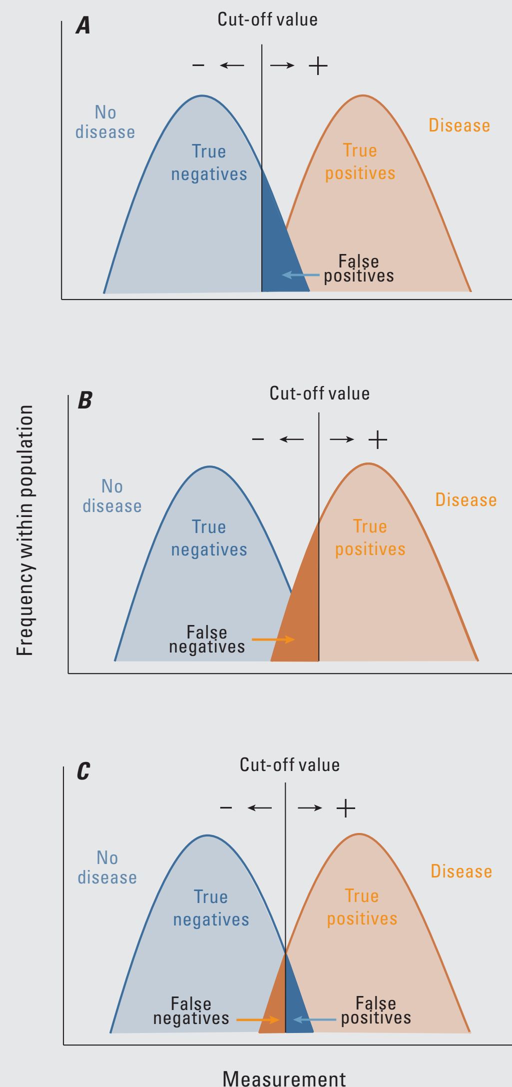


Figure B5.1. Distributions of diseased and nondiseased individuals within a population based on the measurement result of a diagnostic screening test.

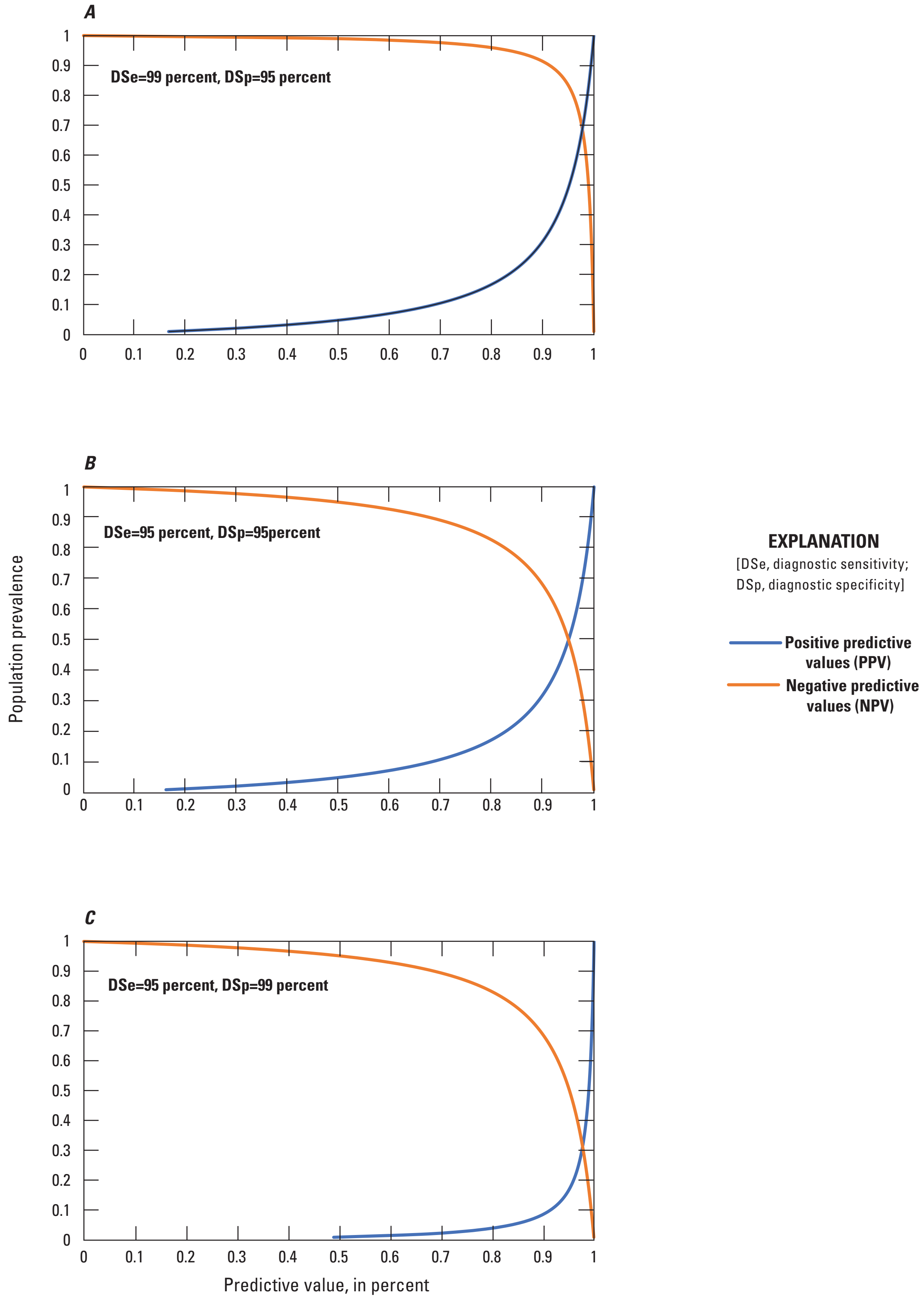


Figure 13. Hypothetical positive predictive values and negative predictive values for a range of population prevalence estimates from 0 to 100 percent.

Accuracy and trueness provide a measure of how well a diagnostic test achieves its intended purpose. Understanding that purpose is therefore critical when evaluating the significance and potential cause of discordant results (in other words, false positives or false negatives). For assays used to detect a pathogen (not disease) discordance cannot result from a natural overlap in the range of test values because the target organism cannot be simultaneously present and absent. Rather, in this context, discordance indicates errors or interference in the testing procedure itself (Davis and others, 2018). A false negative may arise from poor nucleic acid recovery during extraction, pipetting errors, or failure of the target nucleic acid to amplify because of inhibition or amounts below detectable limit (Yang and Rothman, 2004; Espy and others, 2006; Burd, 2010; table 9). Explanations for false positives include contamination, nonspecific amplification, or cross-reactivity to a closely related organism (Espy and others, 2006; Burd, 2010;table 9). All these possibilities can be greatly reduced, if not eliminated, through careful assay design and optimization, proper sample handling, and appropriate use of controls (Espy and others, 2006; Burd, 2010; Johnson and others, 2013). As with any diagnostic test, unexpected or discordant results should be evaluated within the laboratory to identify and correct potential causes before reporting (table 9; Espy and others, 2006; Dessau and others, 2018).

When the current, widely used *Pd* qPCR assay was first developed (Muller and others, 2013), the fungus was causing large die-offs of bats in hibernacula of the eastern United States. The number of carcasses available provided ample samples from affected bats with which to compare molecular results and histopathology and to confirm the presence of the pathogen and the disease. Assay development included comparison to previously analyzed skin samples (42 without and 49 with histopathologic evidence of WNS) to evaluate concordance (Muller and others, 2013). Complete agreement between the two diagnostic methods implied high diagnostic sensitivity and specificity for the qPCR assay, and there was little need to draw a clear distinction between presence of the fungus and the disease it caused. As *Pd* and WNS have moved across North America, however, surveillance objectives have shifted from detection of WNS (the disease) to detection of *Pd* (the pathogen) and sampling methods have expanded to include environmental swabs and sediment (Lorch and others, 2013b; Verant and others, 2016). The discovery that some bat species and populations seem to carry the fungus without succumbing to disease and a new understanding of *Pd* invasion dynamics by which first detection of the fungus may precede first detection of the disease are additional reasons to maintain a clear distinction between the presence of *Pd* and WNS (Langwig and others, 2015a, b; Frick and others, 2017; Hoyt and others, 2020). Recent iterations of both the current WNS case definition (WNS DxWG, 2021) and the National WNS Spread map (fig. 2; https://www.whitenosesyndrome.org/where-is-wns) to include this pathogen/disease distinction further highlight its importance in our understanding of WNS epidemiology.

Understanding the accuracy of the *Pd* qPCR assay is critical for laboratories to monitor the precision of their testing through time. One way that accuracy can be assessed on an ongoing basis is with positive controls (WOAH, 2018c). Additionally, proficiency or ring testing (discussed in the next section) is an effective way for participating laboratories within a network to gauge and compare the performance of a diagnostic test across different platforms or protocols (Viljoen and others, 2005; Kralik and Ricchi, 2017; WOAH, 2018c). As part of the effort to harmonize *Pd* qPCR interpretation, the WNS DxWG has implemented voluntary proficiency testing for laboratories within the network. This testing took place in spring 2021 and results will further inform efforts to minimize sources of discordance in result reporting. Guidance bodies such as NAHLN and WOAH that oversee coordination of diagnostics for reportable wildlife diseases (for example, avian influenza, African swine fever, or foot-and-mouth disease) recommend proficiency testing on a regular basis.

Table 9. Summary of potential explanations for discordant results. If the uncertainty cannot be resolved, laboratories should consider the result invalid and additional samples may need to be collected.

Assay result	True (or presumed true) state for molecular pathogen detection assays	
	Pathogen present	**Pathogen absent**
Pathogen detected	True positive	False positive because of • Contamination (in field or laboratory) • Cross-reactivity • Nonspecific amplification.
Pathogen not detected	False negative because of • Poor sample storage/handling • Inhibition • Poor nucleic acid recovery or pathogen levels too low for detection • Pipetting errors	True negative.

Pseudogymnoascus destructans Quantitative Polymerase Chain Reaction Result Interpretation

When *Pd* qPCR Ct results are low (less than or equal to 35), indicating a large amount of target DNA in the original sample, they are typically considered unambiguous, and interpretation is straight-forward. On the other hand, high Ct results can be difficult to interpret for several reasons. First, from a laboratory standpoint, their accuracy is more uncertain because they are more difficult to reproduce. As previously described, the nature of qPCR provides the reasonable assumption that reduced or late amplification indicates small amounts of target in the original sample, which increases stochasticity throughout the entire processing workflow and, inherently, leads to more result variability. Although this variability is a natural and expected feature of low-level detections, it can also raise questions about contamination or other procedural errors that may have led to erroneous amplification.

Small amounts of target in an individual sample can also be the result of sampling where *Pd* is present in small amounts or is unevenly distributed (at either the site or individual level), leading to lower detection probabilities. The solution is careful sampling design to ensure an adequate number of samples are collected at each sampling unit (site and individual) to account for the increased difficulty in detecting scarce amounts of target material. Although this solution typically falls outside the scope of laboratory involvement, inadequate sampling can contribute to laboratory results that are confusing or difficult to interpret. A recent study examining optimal sampling design for eDNA surveys indicates that even using a highly sensitive assay and multiple test replicates, a low probability (5 percent) of capturing eDNA in the original sample meant that between 75 and 125 samples were required to achieve a 90-percent probability of detecting eDNA in at least 1 molecular replicate (Erickson and others, 2019). This means that when the target is rare or otherwise difficult to detect, even duplicate samples may disagree giving the appearance of inaccurate detection.

During the initial development and deployment of the Muller assay for *Pd* surveillance, any amplification within 40 cycles that met quality-assurance standards for appropriate fluorescence curve appearance and control performance were considered positive. When testing primarily took place in sites with corresponding clinical disease or at times of the year when fungal loads were expectedly high, positive results were commonly strong and unambiguous. As WNS spread through North America, surveillance objectives shifted from confirmation to early detection. This shift led to more frequent sampling in areas that were ahead of the geographic disease “front” with no observed mortality or disease and lower environmental fungal loads. In addition, spring and summer sampling has become more common, a time of year when bats have been shown to harbor less fungus on their skin and fur (Langwig and others, 2015a); consequently, high-Ct surveillance results are now much more common, resulting in the need to revisit diagnostic assay performance and interpretation criteria.

Within the worlds of laboratory diagnostic testing (human and veterinary), increasing confidence in the legitimacy of low-level results typically comes down to the following practices, all of which have been discussed, in depth, throughout this handbook: the use and expected performance monitoring of test controls, the adoption of a robust QMS, and the ongoing assessment of assay performance metrics in relation to new modifications or testing contexts. For highly consequential pathogens, diagnostic laboratories and governing bodies will often go beyond these best practices to establish interpretation thresholds that are associated with the point the assay is observed to lose diagnostic consistency to further increase confidence in results that are designated positive. Nonnegative results beyond that threshold are often designated as inconclusive, to acknowledge their validity while also communicating the associated increased uncertainty.

The analytical performance metrics derived from the 2019 interlaboratory testing (described in detail previously) were used by the WNS DxWG to establish clear result categories of positive, negative, and inconclusive based on an agreed-upon Ct value (box 6). These categories were documented in a revised WNS Case Definition to improve interpretation consistency and better communicate the increased diagnostic uncertainty that has come from using the assay in a new epidemiological context (WNS DxWG, 2021). The cut-off threshold for positive results was evaluated in relation to two metrics: analytical sensitivity and repeatability/reproducibility. As noted, estimates of analytical sensitivity were consistent among laboratories indicating similar assay performance despite protocol variations (table 6). Given this assurance, and the fact that prior interpretation confusion seemed most closely related to nonreproducible results, the DxWG chose a Ct cutoff that was likely to perform well in differentiating between results that are consistently reproducible and those that are not (box 6; tables 6 and 8). Nonnegative results above this value are designated as inconclusive, a category that is meant to indicate elevated uncertainty associated with the results and prompt the submitter to collect or use additional information to fully contextualize the individual result and understand its ecological or epidemiological significance.

Finally, in addition to understanding the assumptions underlying diagnostic performance metrics and how test objectives affect their relevance, it also is important to have a clear understanding of uncertainty and potential biases introduced at each step of the extraction and qPCR process. Often this uncertainty is not formally integrated into the final quantification, and the amount of pathogen calculated from the qPCR reaction is assumed to reasonably represent the amount of pathogen in the original sample. When results are unquestionably positive, accounting for these various sources of bias may be less critical than when a sample is negative or inconclusive. In these cases, it can be helpful to understand how

Box 6. *Pseudogymnoascus destructans* Quantitative Polymerase Chain Reaction Result Interpretation—Additional Information

The current version of the white-nose syndrome case definition (White-nose Syndrome Diagnostic Working Group, 2021) defines the following categories for *Pseudogymnoascus* destructans (*Pd*) quantitative polymerase chain reaction results:

- Cycle threshold (Ct) less than or equal to 37 means *Pd* positive
- Ct greater than 37 but less than or equal to 40 means *Pd* inconclusive
- Ct greater than 40 or no amplification means *Pd* negative.

If future testing reveals new information related to *Pd* behavior on the landscape or qPCR assay performance, these designations may be revisited. Members of the WNS Diagnostic Laboratory Network should follow the categories outlined in the most current White-Nose Syndrome case definition when interpreting and reporting results (WNS DxWG, 2021).

Regardless of the exact numbers, interpretation guidelines will likely continue to rely on Ct values to categorize the result qualitatively (positive, negative, or inconclusive). Most laboratories within the network do not routinely quantify the qPCR results by converting the Ct value into either an amount of DNA (in nanograms or femtograms) or an estimated number of target gene copies. Those that do may be able to provide additional information that can be used by decision makers to contextualize a single sample or suite of results, but currently no guidelines exist within the network to convert quantification estimates to qualitative categories. Additionally, laboratories that report quantified results should be aware of when they can do so appropriately and when they can only report qualitatively. The limit of quantification, which is discussed in more detail in this handbook, is the smallest amount of analyte that can be measured with an acceptable degree of variability (often defined as a variation around the mean less than or equal to 35 percent; Forootan and others, 2017). Because of the inherent stochasticity associated with the Poisson distribution, qPCR quantification estimates increase in variability as the samples decrease in DNA concentration. Table B6.1 of hypothetical results illustrates how quantified results above and below the limit of quantification should be reported.

Table B6.1. Hypothetical quantified result reporting.

[Ct, cycle threshold; DNA, deoxyribonucleic acid; NA, not applicable; LOQ, limit of quantification]

Ct value	Number of quantified DNA copies	Qualitative result	LOQ	Information reported
34.21	127.2	Positive	NA	Ct value, DNA copies, qualitative result
34.81	86.16	Positive	NA	Ct value, DNA copies, qualitative result
35.22	66.02	Positive	60 copies	Ct value, DNA copies, qualitative result
35.94	41.21	Positive	NA	Ct value, qualitative result
36.61	26.64	Positive	NA	Ct value, qualitative result
37.35	16.42	Inconclusive	NA	Ct value, qualitative result
38.59	7.29	Inconclusive	NA	Ct value, qualitative result
40.36	2.30	Negative	NA	Ct value, qualitative result

each step in the testing process may contribute to potentially reducing the amount of target nucleic acid detected (Davis and others, 2018; Mosher and others, 2019). This entails investigating potential sources of uncertainty including the nucleic acid recovery efficiency of the extraction procedure and the error rate from subsampling the recovered nucleic acid for amplification (Davis and others, 2018; Mosher and others, 2019; Taylor and others, 2019; Chaudhary and others, 2020). Laboratories should consider whether these sources of bias are substantial enough to be quantified or whether there are alternative ways to acknowledge the uncertainty when communicating results to partners.

The performance metrics described in this section represent the best available methods for evaluating the soundness of a diagnostic assay. If a network laboratory makes in-house changes to an assay, especially one that is used by other network laboratories, it is important for that laboratory to reevaluate the performance metrics of the modified assay, as detailed in this section, to ensure that changes made do not alter the diagnostic soundness of the assay (WOAH, 2018b). For networks in which participation is voluntary and decision making is based on consensus rather than the governing of an oversight agency, commitment to upholding the quality of results and transparency regarding protocol alterations is paramount to harmonization and successful diagnostic interpretation.

Best Management Practices for Laboratory Network Participation

In addition to ensuring consistent practices for collecting samples, processing samples, and interpreting results, practices established by an organized laboratory network can build and maintain collaboration among various institutions and agencies. Overall, the cooperative relationships built through laboratory networks promote confidence in results and facilitate collective and continuous improvements to applicable techniques (WOAH, 2022). The following section describes additional practices successfully used by laboratory networks that have been implemented or discussed by the WNS DxWG.

Case Definitions

Case definitions are critical to consistent and accurate diagnostic testing and reporting. They are used by public, domestic animal, and wildlife health agencies at the regional, national, and international level to “provide uniform criteria for reporting [disease] cases to increase the specificity of reporting and improve the comparability of diseases reported from different geographic areas” (CDC, 1997, p. 2). In the absence of such uniform criteria, different standards for declaring presence of a pathogen or disease may be applied in different areas or situations, making it difficult to accurately assess trends, investigate outbreaks, or make evidence-based decisions (CDC, 1997; APHIS, 2014). The WNS case definition is maintained and updated by the DxWG to reflect the most current knowledge related to diagnostic assay performance and reflects agreed-upon definitions for various case standards related to both presence of the disease and presence of the fungal pathogen. Laboratory network members should report results in accordance with the criteria detailed in the most up-to-date version of the WNS case definition (WNS DxWG, 2021). If submitters request further information or clarification on results, they can be referred to wildlife disease experts such as, State, Provincial, or Federal wildlife veterinarians, WNS national or regional coordinators, or the National Park Service Wildlife Health Branch, who can assist in providing appropriate epidemiologic interpretation based on the broader biological and ecological context for those samples.

Result Verification

A practice adopted by some formal laboratory networks is that of establishing a reference laboratory that is responsible for dispensing standards, for issuing and analyzing proficiency testing, and for using confirmatory testing after initial detection of a designated pathogen by a member laboratory. All wild bird samples that screen positive for the avian influenza A matrix (IAV-MA) gene in a network member laboratory are only considered as preliminary positive for avian influenza, a disease of economic concern, until confirmatory testing is completed by the USDA’s National Veterinary Services Laboratories. Within this structure, National Veterinary Services Laboratories serves as a reference laboratory with final authority to designate and report sample results as positive or negative based on the results of their in-house confirmatory test results. This scenario is structured on the premise that authority for determining the result of a diagnostic test is assigned to the reference laboratory and this arrangement is mandated through a defined regulatory structure. In the realm of wildlife disease response, such centralized governance is generally lacking, and informal arrangements based on collaboration and consensus are applied instead. For the WNS Diagnostic Laboratory Network, verification of laboratory results is approached on an ad hoc and collaborative basis among member laboratories, often in consultation with the USGS National Wildlife Health Center or the Canadian Cooperative Wildlife Health Center. Verification may specifically be warranted and provide increased confidence in laboratory results that represent initial documentation of *Pd* in a new geographic area or on a new bat species.

There are, however, caveats to consider when deciding whether to seek independent verification of a laboratory result. The first consideration is what sample type(s) may be available for confirmatory testing and how various diagnostic outcomes may support or oppose the initial result. Many of

the procedures for extracting *Pd* DNA from a sample fully consume the original sample material, thus making it impossible to repeat nucleic acid extraction. This means that in most cases, it is only possible for another laboratory to repeat the qPCR assay on existing extracted DNA. If the reason for requesting verification is because of concern of potential contamination, but it is unclear at what point in the process the contamination may have happened, retesting the extract will likely not yield useful information. The second consideration, as previously noted, is the relative strength of the initial result. Reduced reproducibility and increased variability of results is common in qPCR when the amount of target analyte is low, and this has been confirmed specifically for the *Pd* qPCR assay via interlaboratory testing (tables 7 and 8). Based on these results, inconclusive samples (Ct greater than 37) should not be sent to other laboratories for independent verification because the results will likely be uninformative and potentially confusing. Rather, if low fungal loads are expected for the location or species being sampled and confirmatory testing is anticipated or desired, duplicate samples should be collected at the time of the survey. Finally, it is important to consider whether the laboratory seeking verification and the laboratory providing verification use compatible protocols for sample storage, extraction, and analysis. If protocols used by the two collaborating laboratories are different and potentially incompatible, then true confirmatory testing cannot be completed. It is important that laboratories, especially within a voluntary network, are transparent about the methods they use, and that differences in protocols be discussed when result verification is sought.

Proficiency Testing

Interlaboratory testing within diagnostic networks can be designed to suit a variety of purposes. Panels designed to evaluate assay analytical sensitivity may not be appropriate for assessing the performance capacity of either laboratory or staff, making it important to define the objectives of testing before panel design (WOAH, 2018g). Proficiency testing is one such common practice of laboratory networks that involves a panel of blind samples and is intended to achieve several goals. At the laboratory level, regular participation in proficiency testing assesses assay consistency across different laboratories and protocols (Wiegers and others, 2003; WOAH, 2018g). At the individual level, testing acts as a QMS check. By evaluating assay performance in the hands of individual diagnosticians, potential discrepancies can be identified for evaluation and correction (Horowitz, 2013). Additionally, proficiency testing builds trust that supports collaborative relationships between network laboratories and that demonstrates the legitimacy of the network to external partners (Wiegers and others, 2003; Miller and others, 2011).

Design of proficiency panels should follow published recommendations. The WOAH recommends including at least 20 samples per panel, 25 percent of which are known test negative and 75 percent of which are known test positive, and that matrix and storage conditions of the samples should be as similar as possible to true samples (WOAH, 2018g; box 7). Panels should encompass the full range of pathogen nucleic acid concentrations expected to be found in diagnostic samples, and stability of provided materials should be verified before distribution to ensure that tests perform as expected (Viljoen and others, 2005; Clinical and Laboratory Standards Institute, 2013). Results are typically analyzed using z-scores or Kappa statistic for agreement (Taverniers and others, 2004; Conraths and Schares, 2006).

Most blinded proficiency panels are constructed and sent to participants from a designated reference laboratory (Conraths and Schares, 2006; Miller and others, 2011). Without a designated reference laboratory to provide proficiency samples and reference materials, as is the case for the WNS Diagnostic Laboratory Network, members would need to appoint a laboratory to assemble and distribute the proficiency panels, receive and analyze the results, and communicate the results to the rest of the group (Miller and others, 2011). This responsibility could be assigned to a single laboratory, or a rotating schedule could be established. Rotating has the advantage of distributing the burden of test organization and implementation throughout the network and maintaining process integrity by giving each member the opportunity to act as reference laboratory. New laboratories wishing to join the WNS Diagnostic Laboratory Network can participate in the first available round of proficiency testing. If none is planned within a year, a proficiency panel can be prepared and provided to the new laboratory by another member of the network, and their results assessed for trueness based on the most recent round of network testing results.

Surge Capacity and Interlaboratory Support

To provide adequate and timely response to outbreaks, laboratory networks would benefit from having a plan to ensure surge capacity; for example, the NAHLN of USDA APHIS has a specific mandate to do rapid diagnostic testing in the event of a disease outbreak that threatens agricultural resources. This mandate includes assurance that funding and personnel will be available to meet increased needs associated with an emergency response. Similarly, the WOAH has established an international network of Collaborating Centers and Reference Laboratories that are capable of participating in an international emergency response with cooperation from the United Nations Food and Agriculture Organization and the WHO. The extensive partnerships among networks of these

Box 7. Proficiency Testing for *Pseudogymnoascus destructans* Quantitative Polymerase Chain Reaction

In the spring of 2021, the White-Nose Syndrome (WNS) Diagnostic Working Group held an initial round of voluntary proficiency testing for laboratories within the WNS Diagnostic Laboratory Network. Eight laboratories participated across the United States and Canada. The blinded panels consisted of 28 samples that were created at the U.S. Geological Survey National Wildlife Health Center using stocks of conidia (1,000 conidia per microliter [µL]) suspended in phosphate-buffered saline (PBS) and stored in an ultralow freezer at −80 degrees Celsius (°C). The stock suspension was serially diluted using nuclease-free water to the concentrations in table B7.1, and 150 µL was aliquoted into O-ring, screw-cap tubes containing a sterile swab tip to realistically simulate surveillance samples.

Eight negative samples were included that contained only a swab tip and 150 µL of nuclease-free water. All samples were maintained on ice during creation of the panels, which was done in a biological safety cabinet that had been disinfected with 10-percent bleach followed by 70-percent ethanol alcohol before beginning work. Negative controls for all kits were prepared within the hood before removing the conidia solution from the freezer, and additional tubes containing nuclease-free water were left open within the cabinet during kit assembly to detect potential splash contamination. When all samples had been aliquoted, they were labeled with a unique identifying number for each laboratory and sorted based on a key that had been produced in Microsoft Excel using a random number generator.

Kits were returned to the ultralow freezer and stored at −80 °C before shipment. Each kit was shipped to the participating laboratory via overnight service using a Styrofoam shipping box filled with dry ice. Confirmation of receipt and condition was requested and received from each laboratory who were instructed to hold the samples as they would *Pseudogymnoascus destructans* surveillance samples until testing. All data collected for this study are available in a U.S. Geological Survey data release (Alger and others, 2023) and results were communicated with participating laboratories. In addition to providing helpful information to further assess and address sources of interlaboratory variation, the initial 2021 round of testing has laid the groundwork for the continuity of this important practice within the WNS Diagnostic Laboratory Network.

Table B7.1. 2021 proficiency testing panel composition.

Number of samples	Conidia per microliter
5	100
5	10
5	1
5	0.1
8	0

three large international organizations provide considerable leverage for emergency response in the absence of a single funding stream.

The formally regulated networks of the NAHLN, WOAH, United Nations Food and Agriculture Organization, and WHO are typically only activated in response to pathogens that threaten agriculture, domestic animals, or human health. For diagnostic networks concerned with diseases that exclusively infect wildlife, adequate resources and partnerships can be more difficult to secure. Careful planning and communication are needed to ensure that networks devoted to wildlife health have the capacity to respond to emerging or reemerging epizootics (Church and Naugler, 2019). This includes regular assessment of the capacities that each network laboratory possesses for sample storage, sample processing, and high-throughput testing. It may also be advantageous for a network to proactively explore potential funding sources and work with larger agencies to obtain ongoing financial support for interlaboratory testing, network coordination, or rapid response to a wildlife disease outbreak (Stephen and others, 2018).

Summary

The best practices described in this handbook for the voluntary White-Nose Syndrome (WNS) Diagnostic Laboratory Network are based on standards established by other networks with more formal systems of oversight (specifically, the National Animal Health Laboratory Network, the American Association of Veterinary Laboratory Diagnosticians, and the World Organisation for Animal Health). This handbook represents an effort to align voluntary practices with the scientific

rigor that guides regulatory agencies to develop guidelines for the WNS Diagnostic Network to promote provision of the highest quality diagnostic data to wildlife managers and decision makers. Although none of the practices outlined in this handbook are binding, their development and adoption was achieved by consensus within the WNS Diagnostic Working Group (DxWG). Any variation in practice from these recommendations by a network laboratory should be discussed with the DxWG to determine potential effect(s) on diagnostic results and interpretations, and whether additional performance testing may be appropriate.

The WNS DxWG was formed to facilitate the production of accurate and high-quality diagnostic testing for WNS and the causative fungus, *Pseudogymnoascus destructans* (*Pd*). Other critical objectives for the group include providing technical assistance and training tools to partners and collaborating with the WNS Steering Committee to shape strategies for communicating consistent and accurate diagnostic results to natural resource managers and the public. This handbook represents another step towards achieving these goals by outlining harmonized practices for the consistent performance and interpretation of the *Pd* quantitative polymerase chain reaction assay. The topics covered in this handbook provide an important framework to evaluate, harmonize, and implement diagnostic methods to ensure timely and accurate identification of *Pd* and WNS by participating laboratories supporting national goals for WNS surveillance.

Diagnostic harmonization is common among laboratories that do diagnostic testing in support of human and domestic animal health but has yet to be widely embraced or applied to the field of wildlife health. Emerging pathogens are an increasing threat to biodiversity and to wildlife species of conservation concern, which highlights the need for accurate and timely detection of wildlife pathogens, regardless of where the testing is done. Diagnostic harmonization promotes integrity and consistency of test results among laboratories participating in a network while also affording flexibility to individual laboratories to implement documented practices consistent with their needs and capabilities. The work documented in this handbook hopefully guides diagnostic approaches for bat WNS that can serve as a model for groups interested in building collaborative networks and for harmonizing diagnostic practices for other wildlife diseases.

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For more information about this publication, contact:
Director, USGS National Wildlife Health Center
6006 Schroeder Road
Madison, WI 53711

For additional information, visit: https://www.usgs.gov/centers/nwhc

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