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PLANT DISEASE FEATURE

A New Era in Federal Quarantine and State Certification Diagnostics at Clean Plant Centers in the United States

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Abstract

Quarantine and certification programs exist to prevent the entry or spread of harmful pests and pathogens into agricultural systems. Their common objective is to identify pathogen-free source material through the application of validated testing methods for subsequent release for propagation. Tests must be accurate, efficient, and cost-effective. In recent decades, the best tests have been biological assays in conjunction with PCR testing. High-throughput sequencing (HTS) has now become a reliable and cost-effective diagnostic method having greater accuracy and efficiency than biological assays. In this article, we review the role of clean plant centers in quarantine and certification programs, as well as the process by which HTS was evaluated as a testing method to replace biological assays for screening source material. The data from this evaluation included a side-by-side comparison of HTS and biological assays for cultivars of grapevine, *Prunus*, and rose and intra- and interlaboratory validations of an HTS protocol. Based on the results of these evaluations, in 2021, the U.S. Department of Agriculture Animal and Plant Health Inspection Service and several state regulatory agencies accepted the use of HTS and quantitative PCR to test new introductions of source material, replacing biological indexing. This new protocol requires testing at two timepoints within at least a 6-month interval and a dormancy separating the two tests. Under ideal conditions, testing can be completed in 18 to 24 months with subsequent release from quarantine of plant material that has tested negative for regulated pathogens. This new testing protocol has a profound impact on quarantine and certification programs, facilitating quicker access of stakeholders to clean materials for propagation and increasing the number of pathogens that are detected, and even discovered, with reduced cost, effort, and time.

Keywords: clean plant center, diagnostics, high-throughput sequencing, quarantine, viruses

The Cost of Regulated Pathogens in Specialty Crops

Specialty crops are defined in the United States as fruits and vegetables, tree nuts, dried fruits, and horticulture and nursery crops, including floriculture (USDA-AMS 2020). Viruses, viroids, and phytoplasmas, referred to as regulated pathogens in this article, and the diseases they cause in the perennial specialty crops such as grapevines, *Prunus* (i.e., stone fruit trees almond, apricot, cherry, nectarine, peach, and plum), and rose cause millions of dollars in economic losses in the United States (Fuchs et al. 2021; Yeh et al. 2019). These include revenue losses as a result of lower crop value and sales, increased costs of crop management and disease eradication, barriers to trade, and job losses because of lower crop production. In grapevines, grapevine leafroll–associated viruses cause estimated accumulated losses of US$25,000 to $226,000 per hectare over the 25-year life of a vineyard (Atallah et al. 2012; Ricketts et al. 2015), or $90 million annually in California (Cheon et al. 2020). Grapevine red blotch virus (GRBV) alone causes estimated losses of $2,200/hectare in Washington state to $69,500/hectare in California over the 25-year life of a vineyard (Ricketts et al. 2017). The plum pox virus eradication program in Pennsylvania was estimated to have cost $59 million from 1999 to 2009, with more than 2,500 fruit trees destroyed (Welliver et al. 2014). Little cherry disease, caused by little cherry virus 1 (LChV-1) and LChV-2, and Western X phytoplasma are currently affecting cherry trees in the Pacific Northwest, causing losses of more than $115 million since 2015 (Clarke et al. 2024). The annual cost of rose mosaic virus, which causes serious disease in roses, is $28 million to $85 million (Ribera et al. 2020), and the annual loss of rose sales as a result of rose rosette virus, which ultimately kills roses, is estimated to range between $5 and $10 million (Windham et al. 2023). The economic impacts of these diseases highlight the need for effective management strategies.

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Disease Management of Regulated Pathogens

Unlike for fungal and bacterial infections, there are no chemicals that can be sprayed on plants to kill graft-transmissible pathogens or protect plants from infection. Once a plant is infected, it stays infected. Therefore, effective control measures focus on preventing plants from becoming infected in the first place. Although pathogens can also be transmitted to noninfected plants by insects, pollen, and nematodes, clonal propagation and grafting are major modes of pathogen transmission in perennial specialty crops if unverified cuttings and buds are used as source material. Most perennial specialty crops are generated by clonal propagation, which is the process of creating plants asexually from a vegetative plant part rather than from true seed (Fig. 1). Clonal propagation is advantageous because it creates genetically identical progeny that maintain the desirable traits of the parent and can produce many plants from a single individual in a short period. Unfortunately, if regulated pathogens are present in the cuttings or buds used for propagation, they will be passed on to the progeny, possibly causing disease and serving as a source of pathogen that could be disseminated by mechanical means, pollen, or vectors in the field.

Clean plant centers and the National Clean Plant Network

It is essential to establish foundation planting stock that tests negative for pathogens and to use source material from these stocks for propagation and grafting. In the United States, this planting stock is produced by clean plant centers. Historically, clean plant centers were federally funded via state agricultural experiment stations and land-grant universities, but with reduced federal funding in the early 2000s recession, the future of clean plant centers was unclear. In response, industry members, scientists, and other interested stakeholders came together and in 2008 formed the National Clean Plant Network (NCPN) with the mission of protecting U.S. specialty crops from economic losses as a result of plant pests and diseases (National Clean Plant Network 2024). NCPN operates under, and receives funding from, the U.S. Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS). Today, there are 23 participating clean plant centers in 16 states throughout the United States (Fig. 2). The current NCPN crops include berries, citrus, fruit trees, grapevines, hops, roses, and sweetpotatoes.

The economic returns on plant material from these clean plant centers is an estimated $10 to $117 to growers for every dollar invested in clean grape centers (Fuller et al. 2019; Troendle 2017). The most recent study of returns of grape clean plant centers concluded a benefit-cost ratio of $22:1 to $96:1 for California vineyards using leafroll virus-tested grapevines (Li et al. 2022). An evaluation of the program for fruit trees concluded that $420 was gained per dollar spent on clean plant centers if the benefits to growers, nurseries, and consumers were combined (Cembali et al. 2003).

The journey from source material to tested foundation material

NCPN clean plant centers introduce plant material from either international or domestic sources, often referred to as new introductions, and test it for regulated pathogens of concern, as shown in Figure 3. If detected, the source material undergoes treatment to eliminate these pathogens (Fig. 3).

Various pathogen elimination techniques have been developed, but microshoot tip tissue culture is the most reliable and widely used method. A growing microshoot tip less than 0.5 mm is excised aseptically and used to generate new plants using tissue culture methods (Fig. 4). These tissue culture plants, in addition to source material that did not require pathogen elimination therapy, go through a testing protocol. Material that is negative for regulated pathogens of concern is released as provisional foundation stock, and once tested to confirm that the variety is true to type, it is planted in foundation blocks and greenhouses as generation 1 (G1) plants (Fig. 3).

Because clean plant centers produce and maintain a limited amount of G1 material, it is distributed to nurseries for propagation and maintenance in registered increase blocks (G2 to G4; Figs. 4 and 5). Cuttings taken from registered increase blocks are called registered stock, and plants propagated from this registered stock can be sold to growers as certified planting material, if it meets state certification standards (Fig. 5).

Federal and state regulatory agencies

Although NCPN clean plant centers create G1 material that is increased into millions of certified plants by nurseries, the process by which the material is received, tested, released, and maintained is regulated by federal and state agencies. In the United States, USDA-APHIS regulates quarantined source material imported from outside the United States via controlled import permits. APHIS determines which pathogens are agronomically significant and therefore require diagnostic testing and which testing methods and

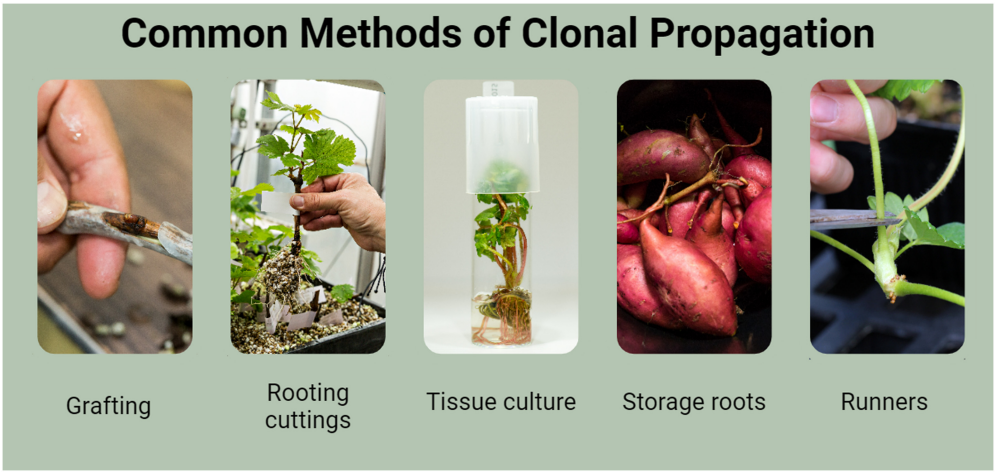

Fig. 1. Common methods of clonal propagation used in specialty crops. Grafting is commonly used to propagate grapevines and trees, whereas rooting cuttings are used to propagate roses but can also be used for grapevines. Tissue culture can be used to propagate all specialty crops, but storage roots are primarily used to propagate sweetpotatoes and runners are used to propagate strawberries. Although clonal propagation is advantageous for generating genetically identical progeny, it can also be a conduit for pathogen spread.

timeframes are acceptable. To obtain an import permit, clean plant centers must submit detailed standard operating procedures (SOPs) for all activities related to handling quarantine plant material for propagation purposes. These activities include receiving and inspecting imported plant material, constructing quarantine facilities to restrict the entry or exit of insect vectors, regulating access to quarantine greenhouse and screenhouse facilities, devitalizing plant material waste and the associated soil, diagnostic

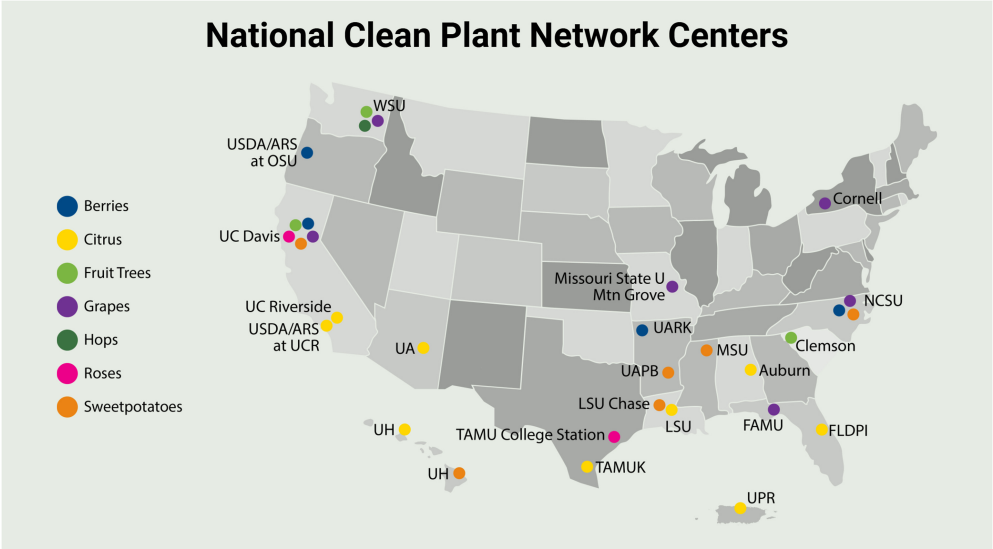

Fig. 2. Clean Plant Centers across the United States are part of the National Clean Plant Network (NCPN). NCPN encompasses seven clonally propagated categories of specialty crops.

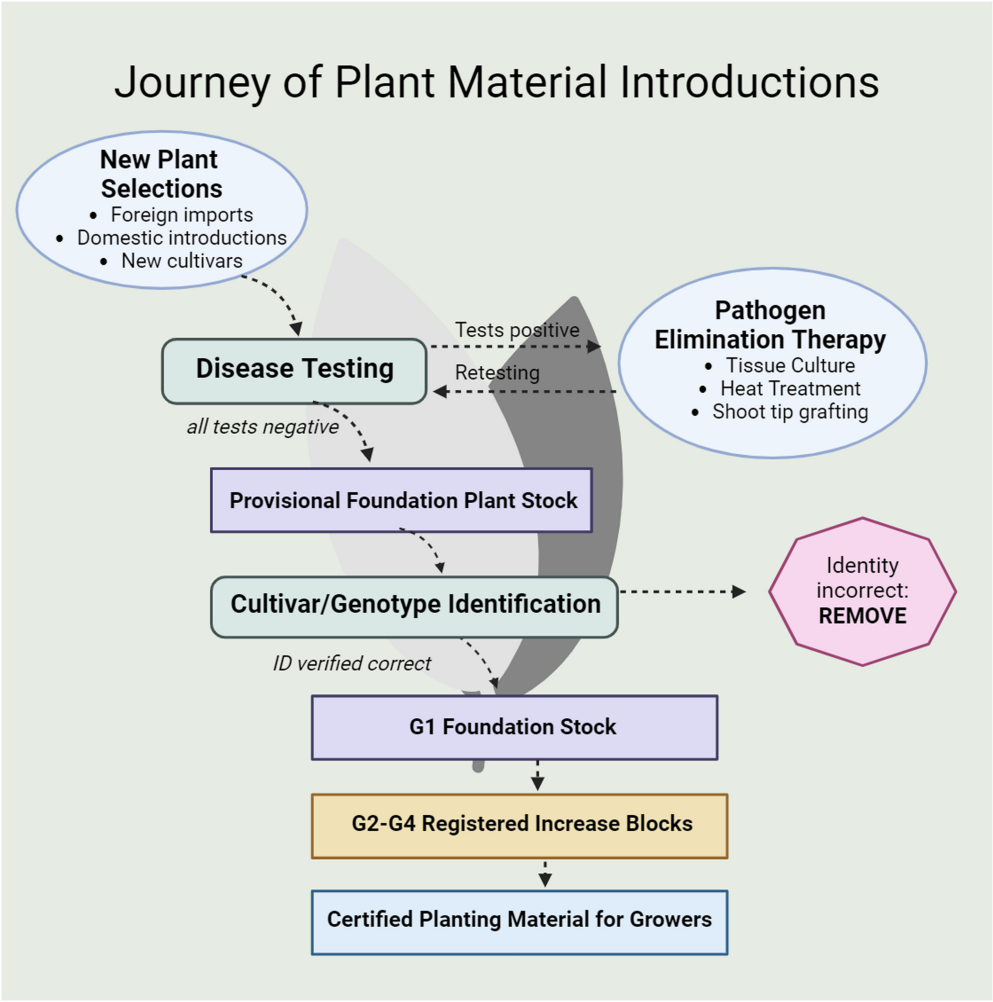

Fig. 3. The journey of plant material introduced at a clean plant center for inclusion in the generation 1 (G1) foundation block.

sampling and testing protocols, and quarantine release requirements. Clean plant centers are also inspected to ensure that facilities are satisfactory and SOPs are followed.

Upon completion of the required testing at the clean plant center, a summary of the results with request for quarantine release is submitted to both USDA-APHIS and the state department of agriculture. Crop selections approved for release may be included in the foundation collections and distributed to stakeholders. Domestic material follows the same process for diagnostic testing but is not regulated by USDA-APHIS.

Once released and planted, the pathogen-tested status of G1 plants is verified by state certification programs and not by NCPN. Most state certification programs follow strict guidelines established by that state's department of agriculture that require routine pest management and testing of G1 plants for regulated pathogens. Nurseries participating in a state certification program maintain registered G2 to G4 increase blocks that are inspected annually and tested as needed by state personnel. State certification programs establish eligibility requirements, planting and maintenance requirements, inspection and testing procedures, and approval,

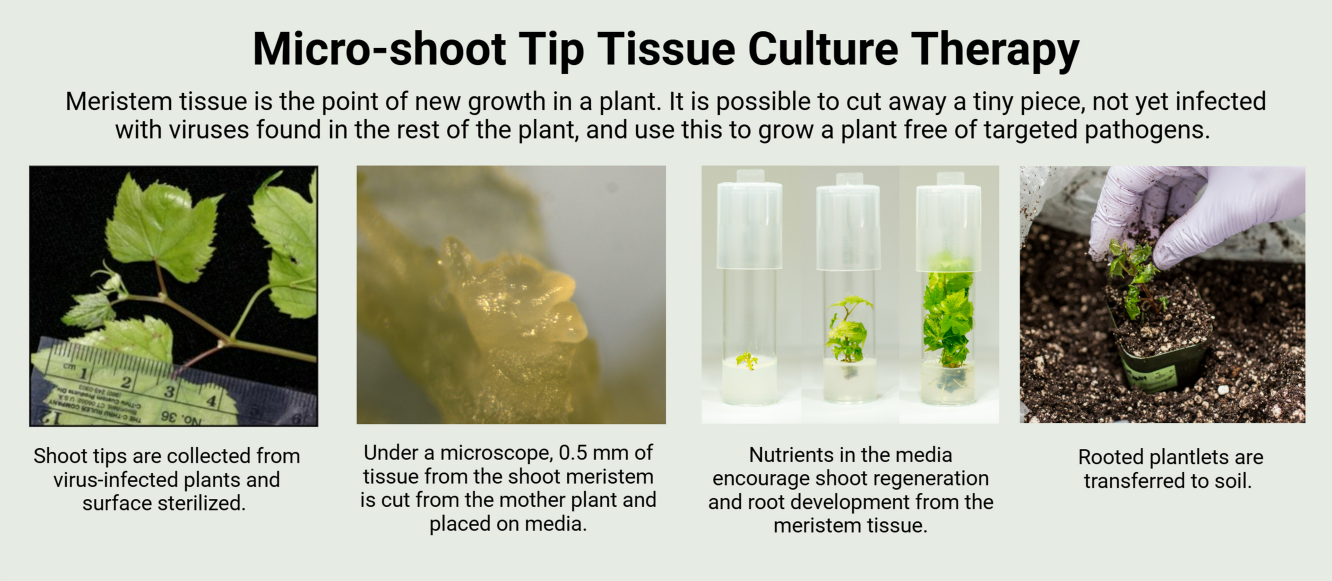

Fig. 4. Microshoot tip tissue culture therapy, as used to propagate a new grapevine plant free of regulated pathogens.

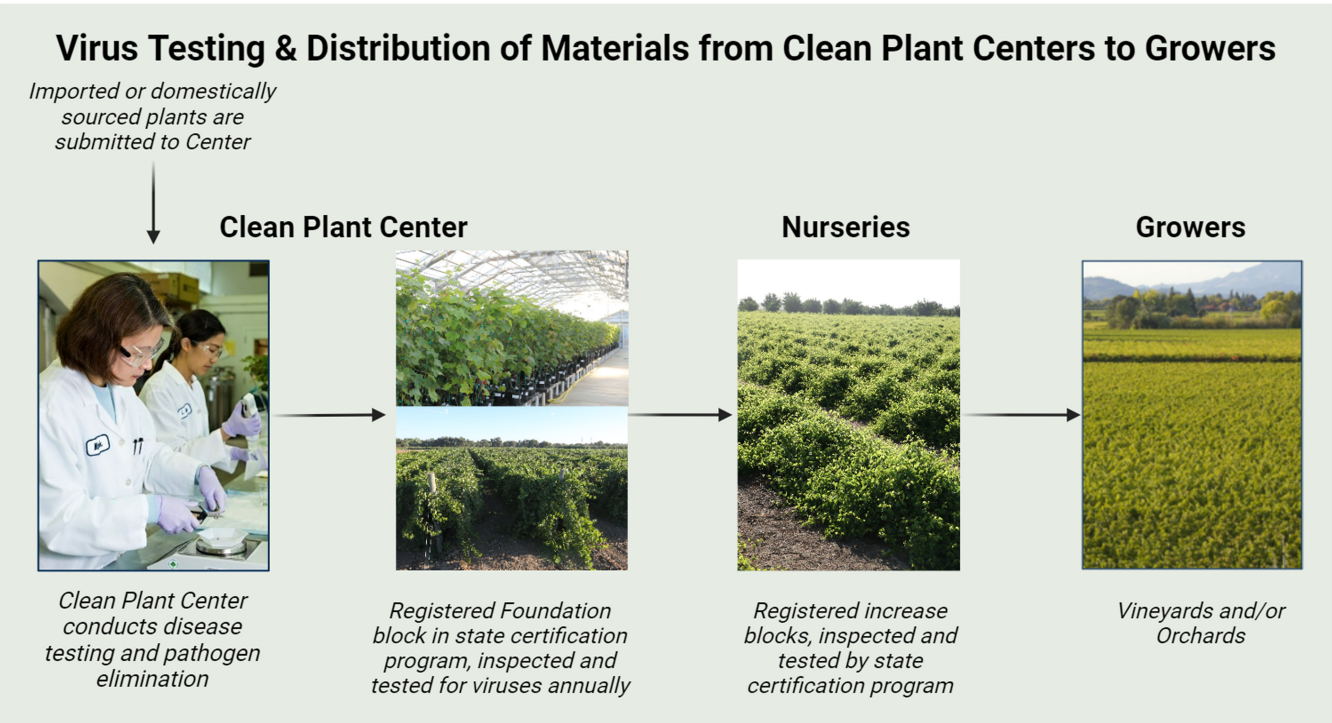

Fig. 5. Plants free of regulated pathogens can be entered into the generation 1 (G1) foundation block at the clean plant center after therapy and testing are completed. G1 material is distributed to nurseries, who in turn grow and test material that they will sell to growers.

suspension, and cancellation of certification. These established procedures help ensure that the commercial quantities of plants from foundation (G1) and increase (G2 to G4) blocks have been thoroughly tested.

Foundation Plant Services—The largest U.S. clean plant center

Foundation Plant Services (FPS) at the University of California, Davis (UCD) in Davis was established as a clean plant and importation center in 1958 and has been an NCPN center since the network was established in 2008. In addition, FPS is the NCPN grapevine and rose headquarters and a regional center for fruit trees, sweetpotatoes, and strawberries. When FPS was founded, California vineyards were experiencing severe virus-related disease problems that were likely introduced inadvertently through virus-infected planting stock. In response, scientists from UCD and the USDA Agricultural Research Service developed methods for the detection and elimination of grapevine viruses. A large collection of pathogen-tested grapevine selections was created, which then became the G1 foundation for the California Grapevine Registration & Certification Program. In addition to being tested, G1 material at FPS is identified using both ampelographic (visual) observations and DNA analysis to ensure it is true to variety. As of September 2024, the FPS collection includes selections of 2,459 grapevines, 532 fruit and nut trees, 1,003 roses, 32 strawberry, 8 pistachios, 54 sweetpotatoes, and 34 olives (Fig. 6). Across the seven plant collections, more than 100 new selections are added each year. Today, FPS is the primary source of high-quality, true-to-variety, virus-tested planting material for nurseries in California and beyond. Looking at nursery sales for grapevines alone, 20 to 70 million grapevines sold each year can be traced back to FPS G1 material.

Diagnostic testing methods in quarantine and certification programs: A historical account

An accurate and reliable detection of regulated pathogens is key for the operational success of a clean plant center. Until recently, source plants entering most clean plant centers, including FPS, were tested by biological assays and either a serological or a molecular assay such as PCR. Because PCR has largely replaced serological methods, only PCR will be discussed further.

Biological assays, referred to as woody and herbaceous indexing, have long been employed to detect the presence of pathogens through disease symptom development. Biological assays are based on the ability of certain plants, called indicator plants, to produce disease symptoms when inoculated with infectious agents such as virus, viroid, or phytoplasma. Inoculation can occur mechanically by rubbing leaves of healthy indicator plants with plant sap from the source material or by grafting buds from source plants onto the indicator (Fig. 7). The former is called mechanical inoculation, which only works with mechanically transmissible viruses, whereas the latter is referred to as graft inoculation.

Once inoculated, the movement of pathogens from the source plant into the indicator allows detection through disease symptom development. Before the advent of PCR, biological indicators were the most sensitive test available, but their accuracy is affected by several factors. Symptom expression can be difficult to score accurately and may be affected by the environment, leading to false test results (Constable et al. 2013). In addition, the presence of symptoms on an indicator plant does not definitively identify the causal agent because multiple agents can cause similar symptoms, as is the case of leaf reddening in red-fruited grapevines seen in response to both GRBV and several grapevine leafroll-associated viruses (Al Rwahnih et al. 2013). It is also possible that an indicator may show disease-like symptoms owing to abiotic factors such as plant stress that are not the result of an infection. Additionally, biological indexing is fraught with several logistical disadvantages. Multiple varieties of woody indicators are needed to match susceptible varieties to targeted graft-transmissible pathogens, and not every pathogen has a susceptible indicator host. Pathogen transmission is not always successful, either because of the failure of an infected bud chip to survive or by inadvertently selecting uninfected buds for grafting that are a result of the uneven distribution of pathogens in the source plant. Woody indexing is costly in terms of time, space, and management of resources. Woody indexing for *Prunus* can be done in a greenhouse and completed in 3 to 6 months, but grapevine and rose indexing is completed outdoors and requires

Fig. 6. The collections managed at Foundation Plant Services (FPS) (as of September 2024).

18 to 24 months. The presence of woody indicators in the field also carries the risk of establishing a source of pathogens in the environment that could be transmitted to nearby uninfected crops by vectors such as insects and nematodes.

Because numerous factors can affect the results of biological assays, PCR or quantitative PCR (qPCR) is used in conjunction with biological assays to test source material for a range of known viruses. PCR is a highly sensitive and specific molecular test based on the in vitro amplification of fragments of virus genomes using virus-specific primers. The sensitivity and specificity of virus detection by PCR can be further increased with qPCR, a quantitative method of PCR that uses either a fluorescent dye or a fluorescent-labeled probe to track amplification in real time (Fig. 8). High sensitivity and specificity are advantageous for detecting low-titer infections and distinguishing between closely related viruses, respectively. However, high specificity may lead to false negatives if genetically diverse isolates are present in samples. In addition, prior sequence knowledge is required for primer and probe design, which precludes the detection of new viruses.

High-throughput sequencing

More recently, high-throughput sequencing (HTS) has revolutionized plant virus diagnostics by offering the ability to identify both known and unknown viruses across many different sample types in a single assay without prior knowledge of their nucleotide sequence. HTS makes it possible to sequence hundreds of millions of DNA fragments simultaneously, in contrast to low-throughput technologies that sequence only one DNA molecule at a time. To do this, DNA molecules are cut into smaller pieces, which allows parallel sequencing of millions of DNA molecules simultaneously. Computational analyses then stitch the short stretches of sequences back together by matching overlapping sequences, called de novo genome assembly, or by using a standard virus genome as a reference, called pathoscope read mapping (Fig. 9) after Hong et al. (2014). The outcome is a large data set that includes the exact sequences of all viruses present in the sample, including previously unidentified viruses, known viruses, and diverse virus strains/variants, all in a short time and at a relatively low cost.

The first use of HTS for virus detection in grapevines at FPS was in 2007, when the relatively new technology was used to characterize the virus sequences present in Syrah grapevines with decline symptoms (Al Rwahnih et al. 2009). HTS was also used to resolve the etiology of a graft-transmissible red leaf disease in grapevine. The red leaf symptomatic vines tested negative by PCR for grapevine leafroll-associated viruses, and the cause of symptoms remained a mystery until GRBV was discovered by HTS (Al Rwahnih et al. 2013; Calvi 2011). Since then, FPS researchers have discovered, or supported the discovery of, 35 novel viruses or viral agents using HTS (Supplementary Table S1). Other research groups employing HTS around the world have discovered several other viruses.

In 2012, FPS began to compare HTS and the biological assays used in its quarantine and certification programs to determine whether HTS might be a better-performing method for detecting both known and novel viruses. For the initial comparison, 15 grapevine accessions known to be infected with viruses of regulatory significance to the grape industry were simultaneously screened using both

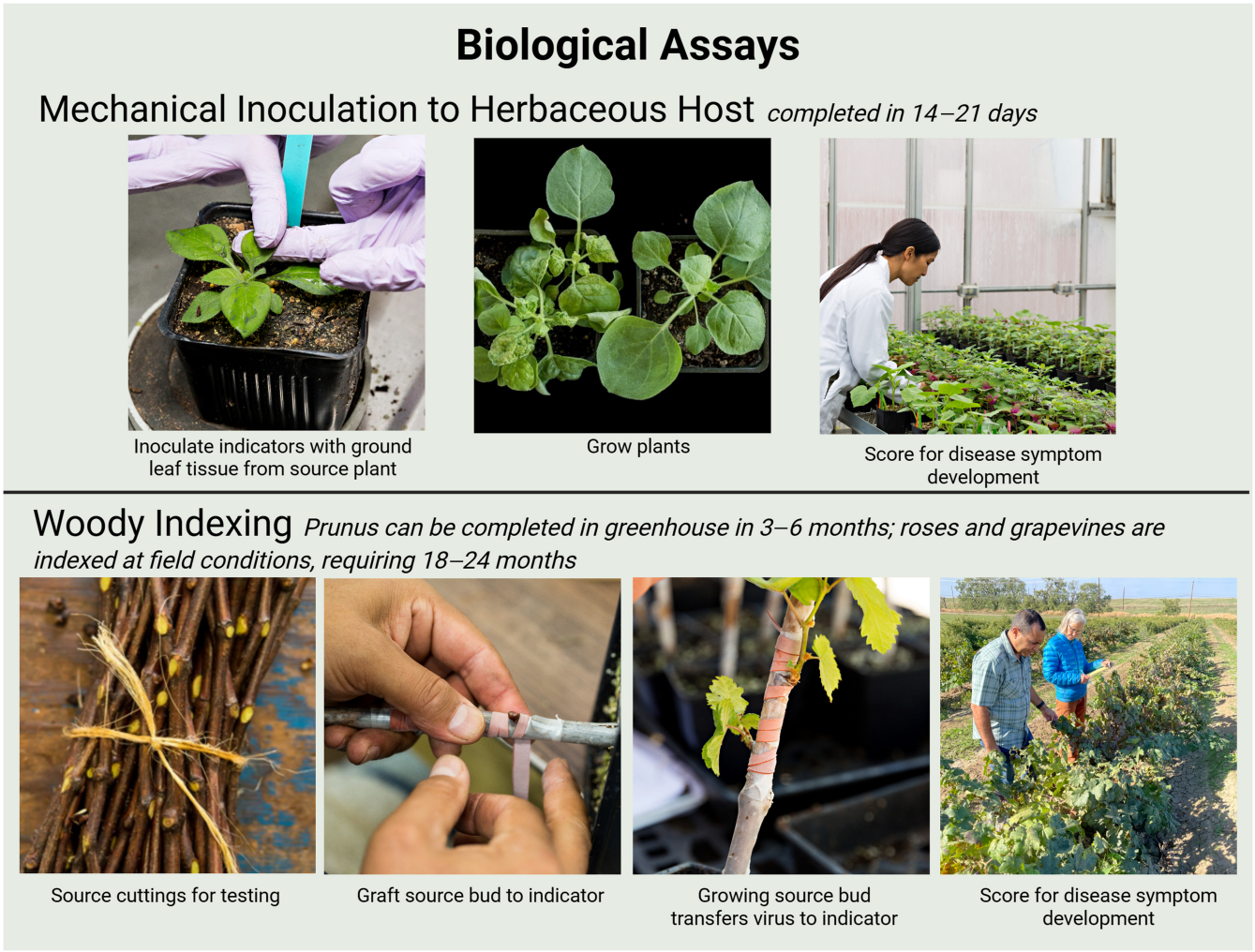

Fig. 7. Two biological assays frequently used by plant quarantine and certification programs.

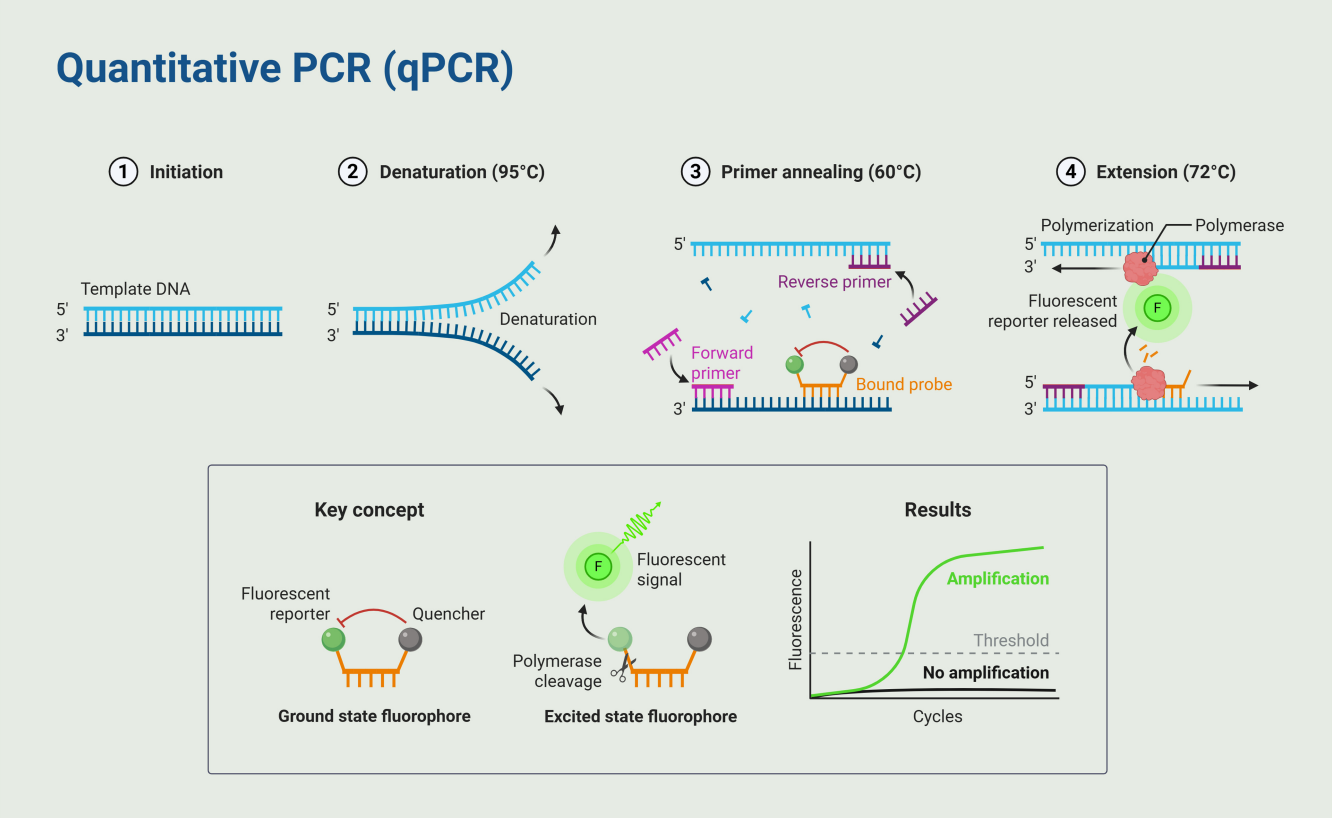

Fig. 8. Quantitative PCR, which can be used to detect viruses in plants. Figure created in BioRender, https://BioRender.com/ix6fxg1

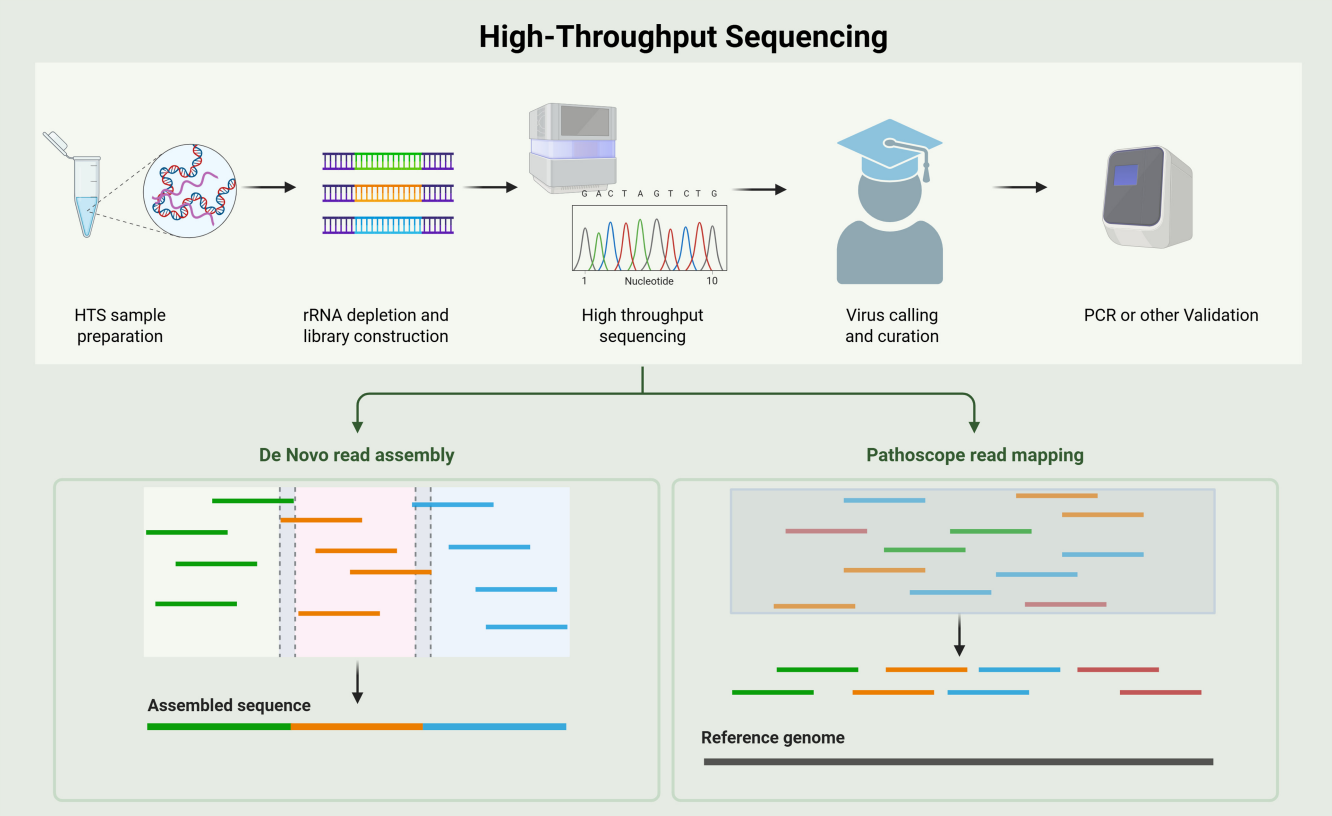

Fig. 9. High-throughput sequencing (HTS) with two complementary bioinformatic analyses as implemented at Foundation Plant Services (Stevens and Al Rwahnih 2024). Figure created in BioRender, https://BioRender.com/cbfz2k5

biological assays and HTS (Al Rwahnih et al. 2015). All viruses detected in the biological assays were also detected by HTS. However, the biological assays failed to detect infections identified by HTS in 8.3% of the indicators, revealing that it was a less sensitive test. In addition, HTS had higher specificity, identifying the specific grapevine leafroll-associated virus, whereas the indicator host only identified the generic leafroll disease.

FPS continued to use biological assays as well as qPCR from 2015 until 2021, as specified by regulations from USDA-APHIS and the California Department of Food and Agriculture (CDFA) for the release of plant introductions but expanded the comparison of HTS and biological assays to include all grapevines, *Prunus*, and roses that were introduced into its quarantine and certification programs. The most significant distinction between the two methods was in the time to completion. The biological assays required nearly 2 years to complete under California conditions or even longer in cooler climates, whereas HTS was completed in weeks. This represents significant savings of not only time but also costs. We estimate that biological assays cost approximately $600 per sample for grapevines because of the high costs associated with grafting and maintaining indicator vines, whereas HTS estimated costs per sample are $300.

Similar results have been published for other crops and by other research groups. Comparison of HTS with conventional testing methods in *Malus*, *Prunus*, and *Pyrus* trees demonstrated that HTS was at least equal to conventional methods and was more effective at identifying novel viruses (Rott et al. 2017). Comparison of HTS with standard biological indexing and PCR protocols in *Fragaria*, *Vaccinium*, and *Rubus* berries was completed by Villamor et al. (2022). The study concluded that HTS detected known and novel viruses in the crops, and envisioned a protocol using HTS for virus detection, followed by confirmation using PCR (Villamor et al. 2022). Taken together, these studies of diverse crop species demonstrated that HTS is more sensitive and specific than biological assays.

HTS validation

Because HTS was more sensitive and specific than biological assays, the next step was to validate the HTS assay as a standalone detection method (Soltani et al. 2021; Stevens and Al Rwahnih 2024). In the process of comparing HTS and biological assays, protocols had been established for sample preparation, nucleic acid extraction, library preparation, sequencing, and data analyses, but these processes had not been validated according to the specific performance criteria of sensitivity, specificity, and repeatability among different HTS data sets. In addition, there was a need to further evaluate HTS performance for grapevines when using leaf petioles collected in the spring relative to dormant canes collected in the fall and to test HTS performance with the addition of an internal virus control from endornavirus-containing *Phaseolus vulgaris* cultivar Black Turtle Soup leaf disks (Soltani et al. 2021). Because sensitivity is the most important performance criteria for a diagnostic assay that is being used in plant quarantine and certification programs, only the sensitivity results will be discussed.

For validation purposes, a pool of 19 grapevines that was qPCR verified for a wide range of viruses and viroids were analyzed (Soltani et al. 2021). Out of a combined total of 264 HTS-detected viruses and viroids, 258 were detected as true positives, for an overall sensitivity of 98%. When spring petioles and fall dormant canes were analyzed separately, sensitivity for detecting viruses in fall canes (98%) was slightly higher than for spring petioles (95%). In all cases, however, if the virus was not detected in the spring petiole sample, it was detected in the fall cane sample from the same grapevine, and vice versa. The apparent lack of correlation in the false negatives for spring petioles and fall canes suggests that these false-negative results were the consequences of lower virus titer that could be associated with season and/or tissue. If the two test results are combined for greater sensitivity by counting a positive from either test as a positive for the plant, sensitivity in the study increased to 100% (Soltani et al. 2021).

Because virus titer can be variable, HTS sensitivity was further evaluated by analyzing a dilution series of three infected grapevine samples. Dilutions were made by combining infected plant material with various amounts of healthy plant material. Dilutions ranged from no dilution to containing 1% of infected plant material (Soltani et al. 2021). Three samples for each dilution were sequenced and the results averaged. The average sensitivity for viruses/viroids was 95% with the most notable decrease in sensitivity at the two lowest dilutions, 2 and 1% dropping to 93 and 85%, respectively. Not surprisingly, the use of HTS as a standalone test may result in false-negative test results in the case of low-titer infections (Soltani et al. 2021). Similar work was done in *Prunus* and rose (data not shown). The results of these studies led to the development of a standardized testing protocol, which includes two testing timepoints and the addition of qPCR. Although using two timepoints compensates for titer fluctuations as a result of seasonal variation, the addition of qPCR allows for an independent confirmation of the HTS results.

Interlaboratory validation

To pave the way for the acceptance of HTS in U.S. importation and certification programs, the HTS protocol reliability was determined through interlaboratory tests. Consequently, an interlaboratory validation study using grapevine, apple, and *Prunus* samples was facilitated by the USDA Plant Pathogen Confirmatory Diagnostics Laboratory (PPCDL) to ensure that multiple laboratories could use the same protocol to achieve similar results. Scientists at the PPCDL develop, adapt, and validate diagnostic methods for the detection of regulated plant pathogens, and their involvement was critical for federal recognition of HTS capabilities. The interlaboratory validation received federal support, with funds provided by both NCPN and Plant Protection Act Section 7721 program administered by USDA. Randomized blind petiole and bark scraping samples were lyophilized and distributed by PPCDL to the three participating laboratories: FPS, PPCDL, and USDA-APHIS Plant Germplasm Quarantine Program (PGQP). All three laboratories used the guidelines developed at FPS for total RNA extraction and HTS library preparation, with slight differences based on equipment. Based on previous analyses, only HTS results yielding more than 15 million filtered reads were considered for analysis. Each laboratory applied its own bioinformatic pipelines for data analyses and the results were compared among laboratories to assess sensitivity. All the three participating laboratories achieved the same level of sensitivity for the test samples (Abrahamian et al. 2022). The validation work with PPCDL and PGQP is an example of the strong working relationship that FPS maintains with USDA scientists and regulators, and the support of scientists at both the federal and state levels has been important for the acceptance of HTS as a regulatory testing method. FPS is also engaging with other scientists from around the world to develop specific standards for the use of HTS in plant health diagnostics. These efforts have been recognized and published by the European and Mediterranean Plant Protection Organization, an intergovernmental body that includes nearly every country in the European and Mediterranean region (Lebas et al. 2022).

HTS replaces biological assays for plant material release and certification

Requests to replace biological assays with HTS for plant material release from quarantine and for certification at the federal and state levels were submitted to USDA-APHIS and CDFA, respectively, and the proposed protocol was accepted in 2021 (Foundation Plant Services 2021). For grapevine, rose, and the fruit tree genera *Prunus*, *Malus*, *Pyrus*, and *Cydonia*, the controlled import permits issued to participating NCPN centers, including FPS, were updated to reflect HTS in combination with qPCR as an approved diagnostic method without the requirement of biological assays. The HTS plus qPCR protocol approved by USDA-APHIS and CDFA requires testing of source plant material at two timepoints with at least a 6-month interval and a dormancy period separating the two tests (Fig. 10).

Grapevines require that the two tests are conducted using different plant materials, cambial scrapings from dormant canes and leaf petioles in summer, whereas *Prunus* and rose protocols use leaf petioles at both timepoints. Plants are only tested when optimal tissue

is available, and if propagated plants do not produce significant growth as needed for the testing interval, timepoints are delayed. At each timepoint, plant samples are tested by both HTS and qPCR and any discrepancies are investigated with repeated testing. Under ideal conditions, propagation and testing can be completed in 18 to 24 months. Importantly, HTS testing offers another advantage of provisional release of the material to nurseries under a Controlled Import Permit (CIP) if the quarantined material is free of regulated pathogens at timepoint 1. Operating under the CIP, the nursery can build quarantined inventory in advance of the full release, which fast-tracks new variety market entry.

Although not required for quarantine and certification purposes, FPS added an additional HTS test upon receipt of the source material, referred to as informative HTS (Fig. 10). This elective testing of the source material provides an early indication of the phytosanitary status of new introductions and eliminates the need to delay virus elimination therapy until after timepoint 1 HTS and qPCR results are complete if the source material is positive for a regulated pathogen. This delay can be significant because tissue culture plants must complete one overwintering season before initial testing (timepoint 1) is performed, and two overwintering seasons before final testing (timepoint 2) to ensure that any virus, viroid, or phytoplasma that escapes therapy has reached detectable levels.

Global adoption

The United States was the first country to approve HTS for use in quarantine programs, followed by Australia (Australian Government Department of Agriculture, Fisheries and Forestry 2022). California approved the use of HTS in select certification programs in 2021, and other states are following suit. Most scientists agree on the benefits of HTS compared with biological assays and have presented guidelines for the adoption of HTS in plant diagnostics (Lebas et al. 2022; Massart et al. 2022; Tamisier et al. 2021).

Despite widespread agreement on the benefits of HTS, its adoption in quarantine and certification programs has been slower than expected, most likely because of the challenges of introducing and then standardizing HTS as a testing method. HTS is a multistep process with each step requiring adequate quality control standards. For high sample throughput, automating processes can minimize human error and the fatigue associated with repetitive processes,

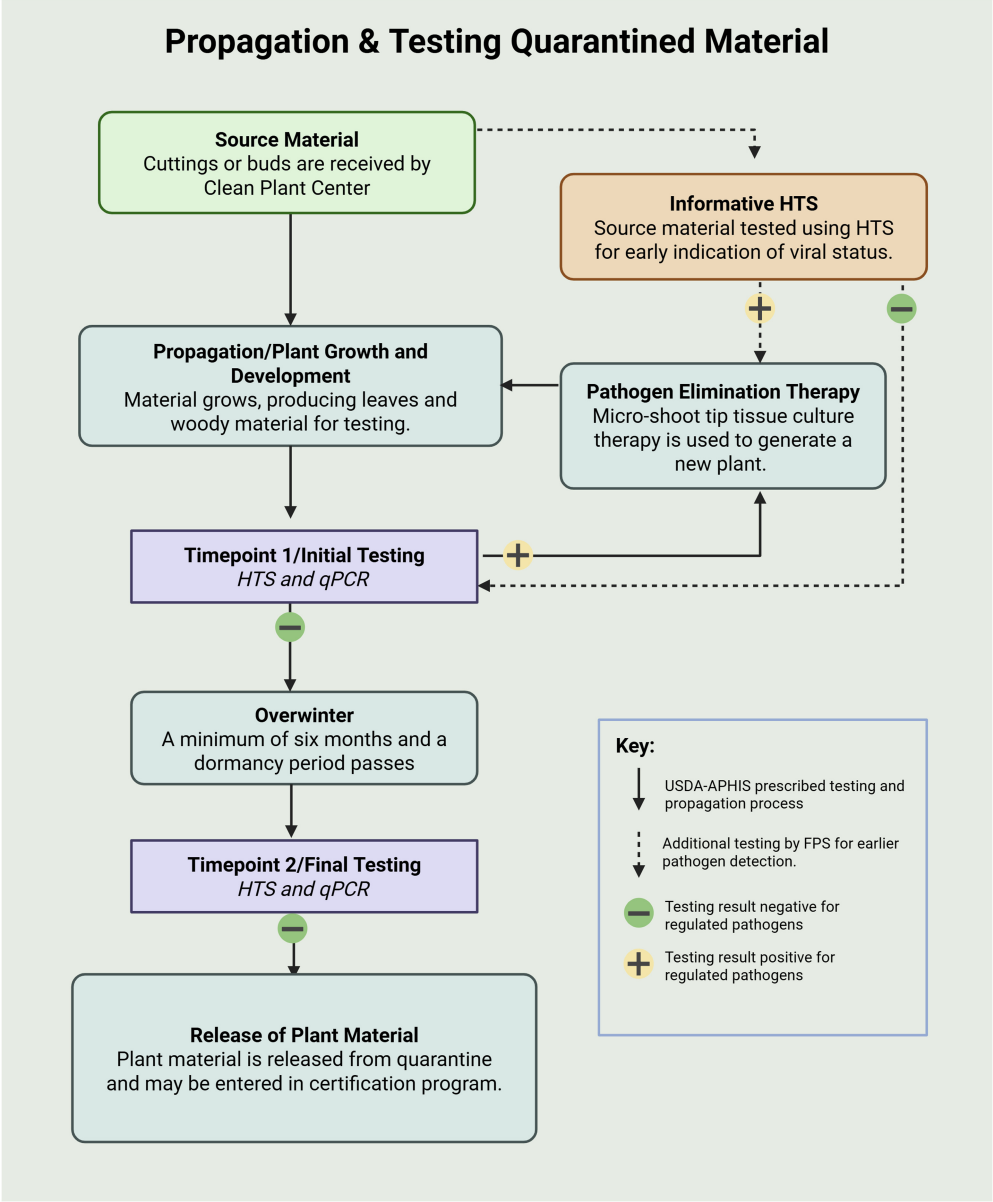

Fig. 10. General high-throughput sequencing (HTS) plus quantitative PCR (qPCR) testing protocol as approved by the U.S. Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS). FPS = Foundation Plant Services.

but this requires capital outlay for equipment. Also, HTS generates large amounts of complex sequence data, which require computational resources, bioinformatics tools, and highly skilled personnel for effective analysis. Finally, once HTS data have been mapped to reference genomes or databases, the results must be reviewed by someone with practical knowledge in plant pathology to determine whether pathogen-like sequences represent real infections or are artifacts.

HTS will continue to reveal new viruses and virus-like agents at an unprecedented rate; hence, regulatory agencies must converge on a systematic approach for reacting to the new discoveries. Otherwise, a lack of adherence to a prescribed process can lead to unjustified restriction on the movement of material and unreasonable testing demands when in fact the identified virus or virus-like agent is not a consequential pathogen based on knowledge of its biology or that of its related taxa. Fontdevila Pareta et al. (2023) present a framework for biological characterization and stakeholder feedback that recommends thorough investigation and regulator involvement before making conclusions about any risk that newly discovered plant viruses and viroids may pose. The four key steps are as follows: confirmation of detection and genome sequence, contextual information gathering and notification to stakeholders, evaluation of association between symptoms and virus presence, and completion of data gaps (Fontdevila Pareta et al. 2023).

Conclusion

HTS, in combination with qPCR, is an important tool in quarantine and certification programs for detecting important viruses, viroids, and phytoplasmas. Compared with biological indexing, previously adjudged as the gold standard, HTS is more sensitive and specific, results are obtained more quickly, and fewer land and greenhouse resources are required. However, because biological assays have been the established gold standard for pathogen detection in quarantine and certification programs for years, a change to HTS plus qPCR requires method validation prior to adoption. When HTS is considered as a replacement for another diagnostic method, it must first be demonstrated as equal or superior to the existing method via a scientific side-by-side comparison. And the results must be reproducible by other laboratories. Only after these criteria are met should HTS be employed in place of another diagnostic method. We hope that as the research advances and literature grows to show the effectiveness of HTS in diagnostics, it will be more widely adopted by quarantine and certification programs. In addition, given the vast amount of data generated by HTS, and the opportunity to detect novel pathogens, we must proceed with caution when determining whether a new virus/viroid should be regulated. This is an opportunity for the scientific community to work together and collaborate with federal and state regulators to determine the significance of these discoveries and balance the impetus to regulate with stakeholder needs.

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Literature Cited

Abrahamian, P., Cai, W., Belanger, C., Nunziata, S., Stevens, K., Hu, X., Hwang, M., Atha, B., III, Yang, Y., Costa, L., Soltani, N., Hurtado-Gonzales, O. P., Al Rwahnih, M., and Rivera, Y. 2022. Inter-laboratory validation studies using high throughput sequencing for detection of viruses and viroids infecting specialty crops. (Abstr.) Phytopathology 112:S3.3.

Al Rwahnih, M., Daubert, S., Golino, D., Islas, C., and Rowhani, A. 2015. Comparison of next-generation sequencing versus biological indexing for the optimal detection of viral pathogens in grapevine. Phytopathology 105:758-763.

Al Rwahnih, M., Daubert, S., Golino, D., and Rowhani, A. 2009. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology 387:395-401.

Al Rwahnih, M., Dave, A., Anderson, M. M., Rowhani, A., Uyemoto, J. K., and Sudarshana, M. R. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. Phytopathology 103:1069-1076.

Atallah, S. S., Gómez, M. I., Fuchs, M. F., and Martinson, T. E. 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. Am. J. Enol. Vitic. 63:73-79.

Australian Government Department of Agriculture, Fisheries and Forestry. 2022. Cutting-Edge Tech Takes Off at PEQ. https://www.agriculture.gov.au/about/news/cutting-edge-tech-takes-off-at-peq (accessed 27 Sep 2024).

Calvi, B. L. 2011. Effects of Red-leaf Disease on Cabernet Sauvignon at the Oakville Experimental Vineyard and Mitigation by Harvest Delay and Crop Adjustment. University of California, Davis, Davis, CA, U.S.A.

Cembali, T., Folwell, R. J., Wandschneider, P., Eastwell, K. C., and Howell, W. E. 2003. Economic implications of a virus prevention program in deciduous tree fruits in the US. Crop Prot. 22:1149-1156.

Cheon, J. Y., Fenton, M., Gjerdseth, E., Wang, Q., Gao, S., Krovetz, H., Lu, L., Shim, L., Williams, N., and Lybbert, T. J. 2020. Heterogeneous benefits of virus screening for grapevines in California. Am. J. Enol. Vitic. 71:231-241.

Clarke, A. E., Catron, K. A., Reyes Corral, C., Marshall, A. T., Adams, C. G., Cooper, W. R., Harper, S. J., Nottingham, L. B., and Northfield, T. D. 2024. *Colladonus* spp. (Hemiptera: Cicadellidae) vectors of X-disease: Biology and management in western United States. J. Integr. Pest Manag. 15:13.

Constable, F. E., Connellan, J., Nicholas, P., and Rodoni, B. C. 2013. The reliability of woody indexing for detection of grapevine virus-associated diseases in three different climatic conditions in Australia. Aust. J. Grape Wine Res. 19:74-80.

Fontdevila Pareta, N., Khalili, M., Maachi, A., Rivarez, M. P. S., Rollin, J., Salavert, F., Temple, C., Aranda, M. A., Boonham, N., Botermans, M., Candresse, T., Fox, A., Hernando, Y., Kutnjak, D., Marais, A., Petter, F., Ravnikar, M., Selmi, I., Tahzima, R., Trontin, C., Wetzel, T., and Massart, S. 2023. Managing the deluge of newly discovered plant viruses and viroids: An optimized scientific and regulatory framework for their characterization and risk analysis. Front. Microbiol. 14:1181562.

Foundation Plant Services. 2021. Foundation Plant Services News. https://fps.ucdavis.edu/newsarticle.cfm?newsid=83 (accessed 3 Oct 2024).

Fuchs, M., Almeyda, C. V., Al Rwahnih, M., Atallah, S. S., Cieniewicz, E. J., Farrar, K., Foote, W. R., Golino, D. A., Gómez, M. I., Harper, S. J., Kelly, M. K., Martin, R. R., Martinson, T., Osman, F. M., Park, K., Scharlau, V., Smith, R., Tzanetakis, I. E., Vidalakis, G., and Welliver, R. 2021. Economic studies reinforce efforts to safeguard specialty crops in the United States. Plant Dis. 105:14-26.

Fuller, K. B., Alston, J. M., and Golino, D. A. 2019. Economic benefits from virus screening: A case study of grapevine leafroll in the north coast of California. Am. J. Enol. Vitic. 70:139-146.

Hong, C., Manimaran, S., Shen, Y., Perez-Rogers, J. F., Byrd, A. L., Castro-Nallar, E., Crandall, K. A., and Johnson, W. E. 2014. PathoScope 2.0: A complete computational framework for strain identification in environmental or clinical sequencing samples. Microbiome 2:33.

Lebas, B. S. M., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G. J., Blouin, A. G., Boonham, N., Candresse, T., Chandelier, A., De Jonghe, K., Fox, A., Gaafar, Y. Z. A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H. J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A., Nakhla, M., Petter, F., Piper, A. M., Ponchart, J., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Roenhorst, J. W., Rollin, J., Saldarelli, P., et al. 2022. Facilitating the adoption of high-throughput sequencing technologies as a plant pest diagnostic test in laboratories: A step-by-step description. EPPO Bull. 52:394-418.

Li, J., Troendle, J., Gómez, M. I., Ifft, J., Golino, D., and Fuchs, M. 2022. Returns to public investments in clean plant centers: A case study of leafroll virus-tested grapevines in support of cost-effective grape production systems. J. Wine Econ. 17:209-224.

Massart, S., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G. J., Blouin, A. G., Boonham, N., Candresse, T., Chandellier, A., De Jonghe, K., Fox, A., Gaafar, Y. Z. A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B. B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H. J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A. G., Nakhla, M., Petter, F., Piper, A. M., Ponchart, J. P., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Botermans, M., Roenhorst, J. W., Rollin, J., et al. 2022. Guidelines for the reliable use of high throughput sequencing technologies to detect plant pathogens and pests. Peer Community J. 2:e62.

National Clean Plant Network (NCPN). 2024. https://www.nationalcleanplantnetwork.org (accessed 27 Sep 2024).

Ribera, L. A., Palma, M. A., Pemberton, B. H., Hall, C. R., Hanselka, D., and Byrne, D. H. 2020. Economic Impacts of the Rose Mosaic Virus. Texas A&M University, College Station, TX, U.S.A. https://agecoext.tamu.edu/wp-content/uploads/2021/05/Economic-Impact-non-RRD-viruses-Roses-1.pdf

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Ricketts, K. D., Gomez, M. I., Atallah, S. S., Fuchs, M. F., Martinson, T. E., Battany, M. C., Bettiga, L. J., Cooper, M. L., Verdegaal, P. S., and Smith, R. J. 2015. Reducing the economic impact of grapevine leafroll disease in California: Identifying optimal disease management strategies. Am. J. Enol. Vitic. 66:138-147.

Ricketts, K. D., Gómez, M. I., Fuchs, M. F., Martinson, T. E., Smith, R. J., Cooper, M. L., Moyer, M. M., and Wise, A. 2017. Mitigating the economic impact of grapevine red blotch: Optimizing disease management strategies in U.S. vineyards. Am. J. Enol. Vitic. 68:127-135.

Rott, M., Xiang, Y., Boyes, I., Belton, M., Saeed, H., Kesanakurti, P., Hayes, S., Lawrence, T., Birch, C., Bhagwat, B., and Rast, H. 2017. Application of next generation sequencing for diagnostic testing of tree fruit viruses and viroids. Plant Dis. 101:1489-1499.

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Soltani, N., Stevens, K. A., Klaassen, V., Hwang, M.-S., Golino, D. A., and Al Rwahnih, M. 2021. Quality assessment and validation of high-throughput sequencing for grapevine virus diagnostics. Viruses 13:1130.

Stevens, K. A., and Al Rwahnih, M. 2024. High-throughput sequencing for the detection of viruses in grapevine: Performance analysis and best practices. Viruses 16:1957.

Tamisier, L., Haegeman, A., Foucart, Y., Fouillien, N., Al Rwahnih, M., Buzkan, N., Candresse, T., Chiumenti, M., De Jonghe, K., Lefebvre, M., Margaria, P., Reynard, J. S., Stevens, K., Kutnjak, D., and Massart, S. 2021. Semi-artificial datasets as a resource for validation of bioinformatics pipelines for plant virus detection. Peer Community J. 1:e53.

Troendle, J. A. 2017. Economic Impacts of Using Virus-tested Grapevines. Thesis. Cornell University, Ithaca, NY, U.S.A.

United States Department of Agriculture, Agriculture Marketing Service (USDA-AMS). 2020. What is a Specialty Crop? https://www.ams.usda.gov/services/grants/scbgp/specialty-crop

Villamor, D. E. V., Keller, K. E., Martin, R. R., and Tzanetakis, I. E. 2022. Comparison of high throughput sequencing to standard protocols for virus detection in berry crops. Plant Dis. 106:518-525.

Welliver, R., Valley, K., Richwine, N., Clement, G., and Albright, D. 2014. Expelling a Plant Pest Invader: The Pennsylvania Plum Pox Eradication Program, A Case Study in Regulatory Cooperation. Pennsylvania Department of Agriculture, Harrisburg, PA, U.S.A.

Windham, M. T., Evans, T., Collins, S., Lake, J. A., Lau, J., Riera-Lizarazu, O., and Byrne, D. H. 2023. Field resistance to rose rosette disease as determined by multi-year evaluations in Tennessee and Delaware. Pathogens 12:439.

Yeh, D. A., Park, K., Gómez, M., and Fuchs, M. 2019. A Review of Economic Studies on Pathogen-tested Plant Materials and Clean Plant Programs for Specialty Crops. Charles H. Dyson School of Applied Economics and Management Cornell University, Ithaca, NY, U.S.A.