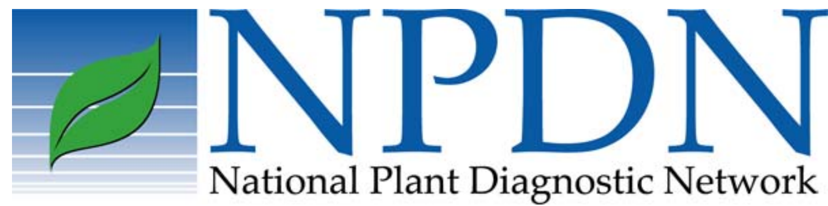
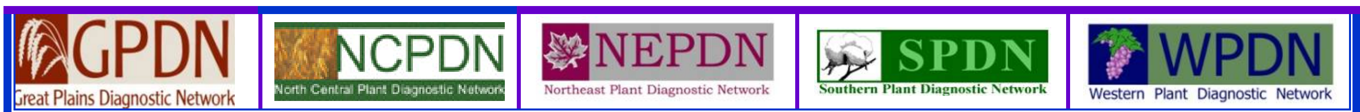


National Plant Diagnostic Network

Standard Operating Procedure for Plant Diagnostic Laboratories

Citrus Greening and the Citrus Psyllid *Candidatus* Liberibacter asiaticus and its vector, *Diaphorina citri*

Version 2.0



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Background: Citrus Greening

This is the NPDN standard operating procedure for plant diagnostic laboratories for the pathogen *Candidatus* Liberibacter asiaticus, the causative agent of citrus greening disease, and its vector. While we will attempt to summarize the current literature, you may find more information at the following websites:

- http://www.sepdn.org/DesktopDefault.aspx?tabid=1033
- http://www.doacs.state.fl.us/pi/enpp/ento/citrusgreening.html
- http://www.crec.ifas.ufl.edu/extension/greening/index.htm
- http://www.eppo.org/QUARANTINE/bacteria/Liberobacter_africanum/LIBESP_ds.pdf
- http://www.aphis.usda.gov/lpa/news/2005/09/greening_ppq.html
- http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=153926
- http://www.tda.state.tx.us/vgn/tda/files/1848/26633_HLBTaskForce.pdf

Impact and history

The pathogens that cause citrus greening can be found on nearly every continent, causing crop and tree loss. Known by several names in Africa and Asia, research has shown that the diseases are all caused by the similar pathogens and are collectively called "greening" now. The disease has been reported since the early 1900s (earlier mention cannot be verified as greening, but reports from India, China, and Taiwan date to the 1890s). Often confused with zinc deficiency, the symptoms include yellow and mottled leaves, fruit that will not ripen, and dieback. Crop losses approach 100% where the pathogen and vector are endemic.

Species

Three species of the pathogen are known today, none of which can be cultured with current methods. Molecular techniques have differentiated the three species of *Ca*. Liberibacter. *Ca*. L. asiaticus vectored by the Asian citrus psyllid and is found primarily in Asia and limited areas in North America. *Ca*. L. africanus is vectored by the African citrus psyllid and is found primarily in Africa. Recently, a third species was reported in South America, *Ca*. L. americanus. All three species cause typical greening symptoms and all are vectored by psyllids.

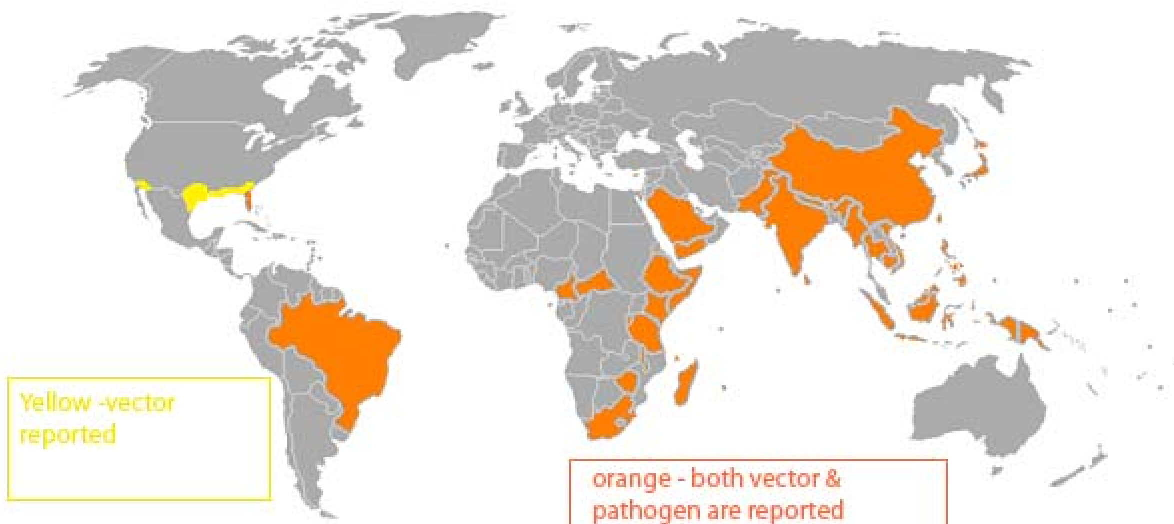
Hosts

All *Citrus* species and cultivars are susceptible, although some are reportedly more tolerant of the infection. Those cultivars and species with some tolerance are still important as disease reservoirs. Other genera reported as hosts include rutaceous plants such as kumquat (*Fortunella* sp.) and *Murraya* spp. In Florida and other southern states, *Murraya* spp. are an important host genus for movement of the disease and the vector, as psyllids find *Murraya* spp. especially attractive.

Transmission

Reports of transmission of the pathogens include grafting, dodder, and adult insect vectors (psyllid feeding).

Useful literature
See Appendix 3



Background: Asian Citrus Psyllid

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae), is an exotic pest species that invaded Florida in 1998 and Texas in 2001. By 2000, the Asian citrus psyllid was detected in at least 31 Florida counties. By in early 2009, the Asian citrus psyllid had been reported in six more states: Alabama, California, Georgia, Louisiana ,Mississippi, and South Carolina. Its accidental introduction into Texas originated from a Florida shipment of potted orange jasmine, *Murraya* spp. Leaf distortions and curling are the typical direct feeding damage symptoms caused by the Asian citrus psyllid. Aphids also may cause somewhat similar damage. However, notched leaves are typical of psyllid damage. Production of honeydew, and subsequently sooty mold growth, is another sign of psyllid infestation, but other related insects, including aphids, scale insects, and whiteflies are also capable of producing this symptom. The most severe damage associated with Asian citrus psyllid is not caused by its direct feeding damage, but by its ability to vector a phloem-limited bacterial disease referred to as citrus greening or huanglongbing (HLB, yellow shoot disease).The other known vector of citrus greening, the African citrus psyllid, *Trioza erytreae* (del Guercio), has not been reported in the Western Hemisphere. All citrus plants and closely related plants in the Rutaceae family including *Murraya, Severinia, Clausena,* and *Fortunella* species are potential hosts for the Asian citrus psyllid. Psyllids appear to prefer Murraya plants on which they can be found most of the year.

The life cycle of the Asian citrus psyllid depends on temperature and food availability, but multiple, overlapping generations are possible. The complete life cycle generally ranges from 15 to 47 days, and each female can lay up to 800 eggs during her lifetime. The life cycle consists of the egg, five nymphal instars, and the adult. Eggs are generally laid on newly emerging shoots or leaves, and this is also the site of most psyllid feeding. Most literature suggests that transovarial transmission does not occur, but this is still somewhat controversial. The Asian citrus psyllid is able to acquire the citrus greening pathogen during 15 to 30 minutes of feeding at the 4th or 5th nymphal instar and the adult life stage. The bacterial pathogen probably continues to multiply within the psyllid once it has been acquired, and the psyllid is believed be capable of transmitting the pathogen to other host plants after 8 to 12 days. Longer feeding intervals increase the probability of transmitting citrus greening to new hosts.

More information (including photographs) on Asian citrus psyllids and citrus greening is available at:

- http://www.sepdn.org/DesktopDefault.aspx?tabid=1033
- http://www.doacs.state.fl.us/pi/chrp/greening/citrusgreening.html

- http://creatures.ifas.ufl.edu/citrus/acpsyllid.htm
- http://ucanr.org/freepubs/docs/8205.pdf
- http://ipm.ifas.ufl.edu/agriculture/citrus/asian.shtml
- http://www.pestalert.org/oprDetail.cfm?oprID=343
- http://www.cdfa.ca.gov/phpps/acp/
- http://www.lsuagcenter.com/en/crops_livestock/crops/citrus/How+to+scout+for+the+asian+citrus+psyllid+and+greening+disease.htm
- http://flyaqis.mov.vic.gov.au/padil/D_citri.html

Protocol: Citrus Greening Disease

Take the appropriate steps, listed below, when plant material suspected of containing *Candidatus* Liberibacter asiaticus is submitted to your laboratory.

1-Shipping:

Sample submission may be directly from a grower questioning the cause of symptomatic plants or from regulatory personnel that have reasons for suspecting a possible infection.

1. Suspect plant material must be placed in double zip-top bags and stored in a refrigerator awaiting shipment to a diagnostic facility. The preferred method for shipment is triple packaging, two zip-top bags and an outer container. Tubes should be sealed with tape. The outer shipping container should be an approved cardboard shipping box. The seams of the box should be closed with approved shipping tape.

2. If submitted by regulatory personnel, the inspector will label and complete the appropriate forms. The inspector should record the state, identifier, grower's license number (if applicable), host(s), inspector's initials and the location and date of inspection. If submitted by a state Department of Agriculture, please include the Department of Agriculture designation: HLB -state-XXX. Upon receipt of the sample, this number will be placed in the notes section of the laboratory's database program so that it can be cross referenced with NAPIS.

3. It is suggested that samples be accompanied by a supplementary data sheet indicating the number of hosts present at each site.

4. Samples should be shipped via overnight delivery or hand delivered to the diagnostic facility.

5. Many of the NPDN regions have established overnight shipping accounts that can be used to ship samples to expert labs. Please check with your regional center before forwarding samples.

2-Receipt and Examination:

Upon arrival, contact submitting entity and acknowledge receipt of sample. The suspect plant material should be examined within a certified biological safety cabinet. Any tools, supplies, and miscellaneous materials used during the examination must be separated and placed in sealed plastic bags awaiting sterilization by a certified autoclave. The surface of all materials must be disinfected prior to the removal from the biological safety cabinet.

3-Storage:

While examination and testing is being conducted, suspect plant material and psyllids must be stored in access controlled cabinets and/or refrigerators. Keeping the suspect plant material and/or psyllids for extended periods of time is not recommended. Plant material should be destroyed using the methods described in section #7.Sample Destruction. Sample destruction is recommended within 2 weeks of submission to your facility if no confirmation has been reported.

4-Screening:

Both initial and advanced screening steps are listed below. Initial screening includes screening for the symptomatic tissue. Initial screening will be carried out by triage laboratories. If these facilities cannot perform the initial screening steps, samples can be referred to the appropriate regional center. Upon completion of the initial screening, advanced screening may be required depending upon initial screening results.

Great Plains Region:
Jim Stack
Kansas State University
Department of Plant Pathology
4024 Throckmorton Hall
Manhattan, KS 66506
(785) 532-1385

Western Region:
Melodie Putnam
Oregon State University
OSU Plant Clinic
1089 Cordley Hall
Corvallis, OR 97331-2903

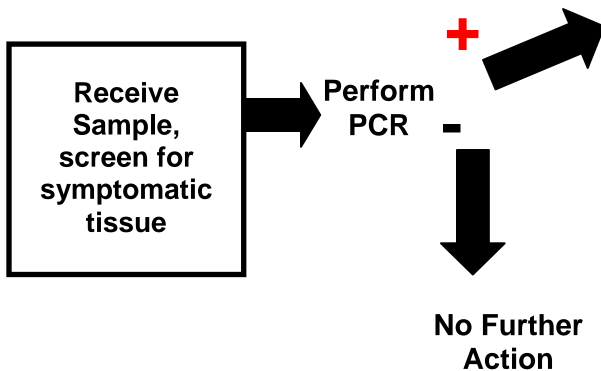
North Central Region:
Jan Byrne
Michigan State University
Diagnostic Services
114 Center for Integrated Plant Systems
East Lansing, MI 48824
(517) 355-3504

Northeast Region:
Karen L. Snover-Clift
Cornell University
Plant Disease Diagnostic Clinic
334 Plant Science Building
Ithaca, NY 14853
(607) 255-7850

Southern Region:
Anne Vitoreli
Plant Disease Clinic
UF Bldg 78 Mowry Road
PO Box 110830
Gainesville, FL 32611-0830
(352) 392-1795
(352) 392-3631 ext 254 (Carrie Harmon)

FDACS Plant Pest Diagnostic Lab:
Tim Schubert
PO Box 147100
Gainesville, FL 32614-7100
(352) 372-3505

Greening Sample Protocol Flowchart



Send DNA extracted from plant tissue to Dr. Mary E. Palm at the PPQ Molecular Diagnostic Laboratory or to a laboratory designated by APHIS-PPQ.

***(If your lab is unable to perform the DNA extraction then please forward the plant sample to your regional expert lab and they will perform the extraction as well as forward the DNA sample.)**

+ = positive result
- = negative result

Initial Screening by Triage Laboratories

1. Screen for symptomatic tissue (see below)

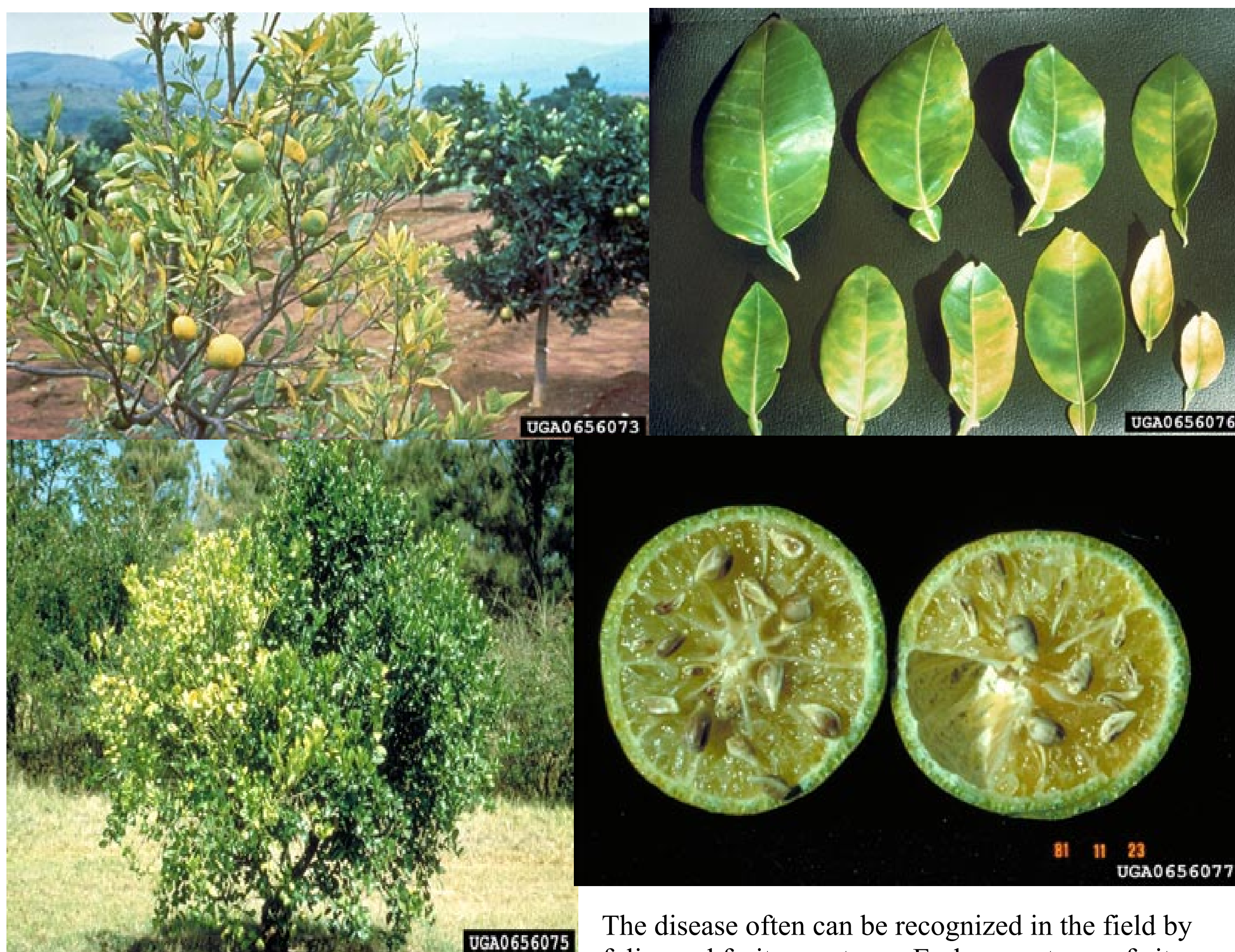
2. Serological Testing
Serological testing is not yet available for this pathogen. Upon release of ELISA or other serological tests, that information will be presented here.

3. Isolation and Morphological Identification of *Candidatus* Liberibacter asiaticus
Isolation of *L. asiaticus* is not possible at this writing. This bacterial pathogen is phloem-limited and no method for culture is presently available.

4. Iodine-based test for starch accumulation. This new test may be used to select tissue for molecular testing. **The full protocol, including pictures, directions for interpretation of results and related tips can be found at the University of Florida Citrus Research and Education Center website:** http://www.crec.ifas.ufl.edu/extension/greening/PDF/HS37500.pdf.

1. Select leaves expressing strong symptoms and avoid those with physical damage or symptoms clearly related to some other problem, e.g. nutrient deficiency. Use of the IFAS Citrus Greening Field ID Pocket Guide or other such tool is helpful.
2. Select symptomatic leaves only from healthy, undamaged branches. Branches that are broken, girdled or are otherwise physically damaged may cause starch to accumulate in leaves even if HLB is not present.
3. Leaves that are in full sun locations are best; try to avoid heavily shaded leaves.
4. Always test at least 2-3 leaves displaying prominent symptoms of HLB.
5. Prepare iodine solution. There are a number of iodine solutions available at drugstores and pharmacies.For this test, purchase products labeled as either "tincture of iodine" or "iodine tincture." These products contain iodine and sodium iodide dissolved in alcohol and water. Other iodine products that are labeled "iodine solution" such as Betadine® (povidone-iodine) contain surfactants and other chemicals that prevent them from reacting with starch. For use in this test, the purchased tincture of iodine should be diluted 1 to 10 with water (i.e. 1 part iodine mixed with 9 parts water). Do *not use* the iodine tincture straight; the undiluted tincture will react very strongly with even small quantities of starch, potentially leading to false positives. The diluted iodine solution should be stored in a dark tinted (e.g. brown glass) or opaque container. Clear containers can be covered with aluminum foil. The prepared solution will last for a few days when properly stored.
6. Using a sharp, clean razor blade, cut a section from the selected leaf that includes the symptomatic tissue. Do not cut through the mid-vein, rather cut sections from the leaf blade on either side of the mid-vein.
7. Immerse the cut section(s) of leaf in the prepared iodine solution for 1.5 - 2 minutes.
8. Remove the sections and rinse with clear water.
9. Examine the cut edge of the section(s) for dark staining using a hand lens or magnifying glass.

Symptoms of Citrus Greening (Photos and text, S. Halbert, J.M. Bové, H.D. Catling Bugwood)



The disease often can be recognized in the field by foliar and fruit symptoms. Early symptoms of citrus greening disease are small yellow leaves on one limb or section of the tree canopy. The most diagnostic symptoms of citrus greening are leaf mottling that often ignores the leaf veins. The newest leaves may show symptoms resembling zinc deficiency, while older leaves have the characteristic greening mottle. Other symptoms are yellow shoots, twig die-back, poor flowering, and stunting. Fruit is small, poorly colored, and/or lopsided. Fruit taste is bitter, medicinal, and sour. Seeds usually abort, and fruit set is poor. Symptoms vary according to cultivar, tree maturity, time of infection, stage of disease, and other abiotic or biotic agents that affect the tree. Chronically infected trees are sparsely foliated and display extensive twig or limb dieback. Although symptoms can provide strong clues to the presence of citrus greening disease, final confirmation by molecular diagnostic tools is needed for regulatory purposes.

Advanced Screening by Expert Laboratory

1. Molecular Identification

Samples will be tested using conventional and real-time PCR. The molecular testing will be done at the USDA-APHIS-PPQ Molecular Diagnostic Laboratory, Dr. Mary E. Palm, Director, or at laboratories designated by APHIS-PPQ. Sufficient plant sample should be shipped along with the extracted DNA to the USDA-APHIS-PPQ. The protocol for sample preparation and DNA extraction for leaf tissue, petioles, and stems is listed below.

If your lab is unable to perform the DNA extraction, please forward the plant sample to your regional hub lab and they will perform the extraction for you as well as forward the DNA sample to Dr. Palm. **Please remember to send the PPQ 391 form with your sample.**

Shipping DNA to Beltsville

- Samples should be collected from symptomatic tissue only because current PCR methods are not efficient in diagnosis from asymptomatic tissue.
- Laboratory personnel should send extracted DNA samples to the address below.
- Label tube caps with permanent fine marker in legible writing.
- Seal tubes with parafilm to prevent sample spilling during shipping.
- Place the labeled, parafilmed tubes in a zip top bag and seal. Place this bag into another sealed zip top bag.
- Finally place both bags into a larger zip top bag containing wet ice. Store samples in a -20 C freezer until package is ready for shipping. **Do not package the sample and 391 forms in the same bag.** All samples should be in one bag and all 391 forms in a separate bag.
- **Email notification is required for all shipments prior to shipping:** Mary.Palm@aphis.usda.gov with cc to Joel.P.Floyd@aphis.usda.gov. This email notification must include the number of DNA samples to be shipped and the FedEx or UPS tracking number. Shipments should be made Monday through Wednesday only.
- Send shipments of DNA and 391 forms to:

Dr. Mary E. Palm
USDA APHIS PPQ Molecular Diagnostic Laboratory
BARC-East, Bldg-580
9901 Powder Mill Rd
Beltsville, MD 20705
Ph 301-504-7154

DNA Extraction Protocols

1. **Plant DNA Extraction Protocol**
   a. **Sample Preparation for DNA Extraction**

   IMPORTANT NOTE: Contamination of PCR samples can start prior to the PCR test during both sample selection and preparation, and DNA extraction.

   To minimize the potential for contamination during the sample selection make sure that the area used in the lab to select leaves is separated from the DNA extraction area. Do not extract DNA on the same bench where PCR products are amplified or analyzed.

   Make sure that disposable lab mats are used on the benches where samples are processed, and samples are cut in weigh boats changed between each sample. Do not cut on cutting boards or the bench directly, as this will result in contamination of the sample. Gloves should be worn to select leaves and cut samples and changed between each sample to

avoid contaminating the following sample. Bacteria and DNA from L. asiaticus-infected leaves can be transferred from infected leaf sets to healthy leaf sets and may result in a false positive PCR test. A good rule of thumb is to treat each sample as if it is infected and as if the next sample to be processed is healthy and take measures to minimize contamination of that next sample. Cutting implements should either be disposed of between samples (autoclave or sterilize prior to disposing of them in a sharps container), or dipped in ethanol and flamed between samples to avoid direct contamination.

Leaf tissue for DNA extraction should be collected from the midrib and petiole of symptomatic leaves (Figures 1 and 2).

- A disposable blade can be used. Dispose of blades, or flame-sterilize the cutting implements between each sample. The midrib of each leaf should be cut out (see Figure 1, below). The maximum amount of tissue used in the DNeasy Plant Mini kit is 100mg (the midribs of approximately 5-6 leaves, each midrib cut into 6-12 pieces).

- The best results have been obtained by cutting out half to two-thirds of the midrib, closest to the petiole, and using just that portion for PCR.

- Make sure to keep leaves and their respective midrib pieces separate, unless pooling the leaves.

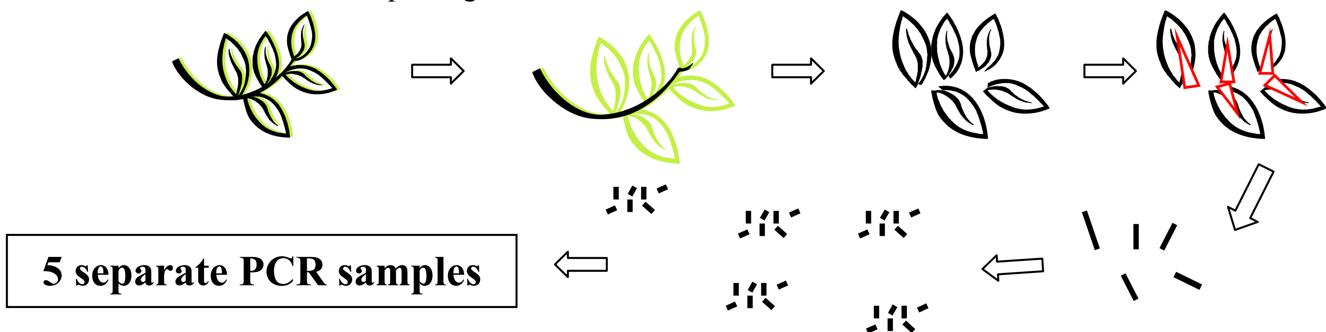


Figure 1. How to sample leaves in preparation for PCR.

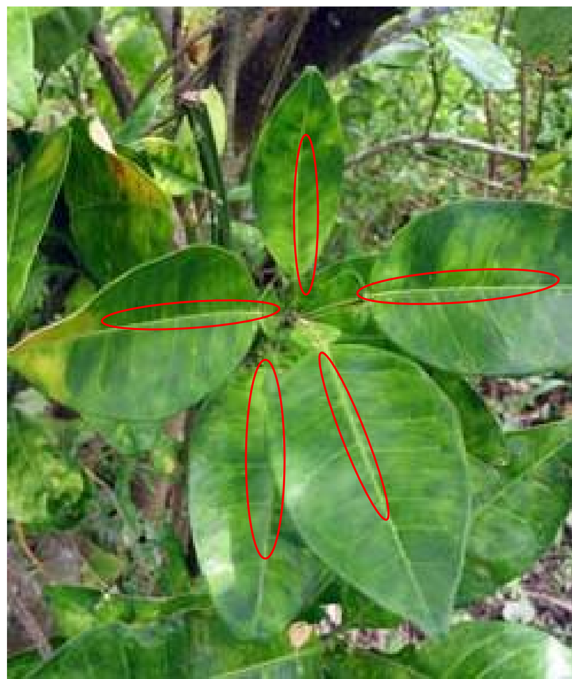


Figure 2. Example of sampled areas from L. asiaticus-infected citrus

b. DNA Extraction

DNA must be extracted using one of the two CPHST validated protocols: the Qiagen DNeasy Plant Mini kit, or the FL Department of Agriculture DNA extraction protocol that finishes with the Qiagen DNeasy Plant DNA kit.

The PCR amplification levels are comparable using both protocols. Using the Qiagen DNeasy Plant Mini kit will also provide a similar quality of DNA for every sample sent forward to the PCR testing lab(s) and will provide consistency in the results obtained.

IMPORTANT NOTES:

- In order to avoid cross contamination, designate a separate room or lab area for the DNA extraction.
- Also, use separate designated sets of pipettes (especially if your lab conducts PCR). Use aerosol-resistant barrier pipet tips.
- Centrifuge any DNA-containing tubes before opening so that any liquid near the rim of the tube is removed; centrifuge rotors designed for aerosol containment are recommended.
- Use microfuge tube openers to avoid contact with the top rim of the microfuge tubes. Your fingers should never touch the top of the microfuge tube.
- It is a good practice to store plant samples or extracts in a separate freezer or freezer compartment from PCR reaction components. If samples are contaminated with soil, rinse them in sterile water and pat-dry them with a paper towel.
- Wear gloves and change them often, particularly between different segments of the DNA extraction procedures.
- Use disposable lab mats to cover bench areas and change them between each set of extractions.
- All racks, tube openers, and other plastic materials used in the procedure should be decontaminated between each set of extractions by soaking in a 10% bleach solution (a 1:10 dilution of commercial bleach in water) for 30 minutes followed by 2 rinses with water to remove the bleach solution. Bench areas, pipettes, centrifuge rotors, lab chairs, drawer handles, and other knobs, etc. in the environment of the bench used to do DNA extractions should be wiped down every couple of days with a DNA elimination solution such as DNA Away (bioexpress.com).

- Samples should remain in a refrigerator or freezer prior to packaging and immediate shipping for PCR testing. Do not have boxes sitting at room temperature for several hours before shipment.

Plant DNA Extracation using Qiagen DNEasy Plant Mini Kit (Cat# 69104)
This protocol is based on Qiagen's protocol for total DNA extraction from plant tissue using the mini columns. A booklet is included with each kit and the protocol is on page 16. Please read carefully all information provided in the booklet before starting work. A DNeasy Plant Extraction Worksheet that provides the protocol and places to record sample information is available in Appendix 4. A list of supplies for this procedure is in Appendix 2.

There have been a few changes made in the protocol noted in the section addressing the extraction protocol. Here are some additional helpful tips:

- Consider extracting as many samples at once as your centrifuge rotor can hold. For example, one person can process 12 samples at one time. Too many samples processed simultaneously can result in some columns beginning to dry before the next step in the procedure.
- Prepare and label all necessary tubes and columns in advance (columns should be labeled on the top of the lid, not on the side of the collection tubes). Place them on a rack in rows:
- First row – the QIAshredder columns (lilac colored, columns supplied)
- Second row – 1.5 ml tubes
- Third row – DNeasy columns (white columns supplied, **make certain the column is labeled since you will move this, not the tube during its use in the procedure**)
- Fourth row – 1.5ml tubes with lids cut off (if necessary).
- Fifth row – 1.5 ml tubes for the DNA extracts clearly labeled with the sample ID and date of extraction.
- Set up a water bath or heating block at 65ºC.
- The first time you use the kit add the appropriate volume of 95-100% ethanol to both the AW and AP3/E buffers. Record on the bottle that EtOH was added and the date added. Always check buffers AP1 and AP3/E for precipitates that can form upon storage. To dissolve the precipitate, warm the buffers up to 65ºC prior to use.
- Wear gloves during extraction. Change them often and if you have any reason to believe they have been contaminated with sample extracts.
- Open tubes and columns carefully to avoid aerosol formation and spills. Use a tube opener. When you transfer samples do not touch the edge of the tubes with the pipet tip.
- When handling columns, please hold them by the top of the collection tube, not by the column. The column sits inside the collection tube. If you hold it by the column only, the collection tube may disconnect and drop (potential contamination or loss of sample).

When transferring columns from one collection tube to another, please do not touch the bottom of the column (this is where the sample will elute and is a potential area to introduce contamination to the sample).

- Once you start the extraction, please do not stop or leave your samples sitting longer than prescribed by the protocol.
- Pre-warm a portion of the AE buffer to 65ºC in water-bath or heating block. (This is from a prior edition of the handbook and how the work instruction was validated)
- Centrifugation steps are carried out at room temperature.
- Most centrifugation steps are to be performed at 20,000 x g or 14,000 rpm. If you do not have a centrifuge capable of that speed, then double the time.

DNeasy Extraction Protocol

- First thing, set up a water bath or heating block to 65ºC.
- Pre-warm a portion of the AE buffer to 65ºC in the water bath or heating block.

(Note: The protocol has been modified slightly from the manufacturers' recommendations.)

1. Select 2-3 symptomatic leaves and excise 100mg of midribs and petioles in a sterile petri dish using a sterile scalpel blade. Cut sample into small pieces and place in a 2 ml screw-cap tube. Flame sterilize blade with ethanol between each sample.

2. Place 3 glass beads (2.5mm diameter) into each tube. Tightly close the cap and flash freeze in liquid nitrogen. Homogenize for 60 seconds using the Mini Bead-Beater. Put tubes on ice while homogenizing the next sample. Repeat homogenization 2 more times. Alternatively, one can use a mortar and pestle to grind samples with liquid nitrogen and then place the ground sample in tubes. Make sure to remove the toweling under the mortar between samples and decontaminate the mortar and pestle, as well as the device used to move the sample from the mortar to the tube, between each grind. (Decontaminate by washing, followed by soaking in a 10% bleach solution for 30 minutes, and finally rinsing well with water to remove the bleach solution).

3. Add 600µ1of buffer AP1 and 6µ1 of RNase A to each tube, mix by vortexing and incubate at 65°C for 10 min. Mix tubes 2-3times by inverting during incubation.

4. Add 195 µ1 of buffer AP2 to each sample, mix well by vortexing and incubate on ice for 5 min.

5. Centrifuge samples for 5 min at full speed (14,000 rpm).

6. Transfer supernatant to QIA shredder spin columns (lilac colored tubes) placed in 2ml collection tubes and centrifuge for 2 min at max. speed.

7. Transfer flow-through fraction into a new 1.5 ml tube (try not to disturb pellets formed at the bottom of the tubes). Usually about 450 µl is recovered. Note volume transferred for next step.
8. Calculate and add 1.5 volumes of AP3/E buffer to each sample (for example, for 450 µl of flow-through add 675 µl of AP3/E). Immediately mix well by carefully pipetting up and down.
9. Pipette 650 ul of sample onto a DNeasy spin column (white columns) and centrifuge for 1 min at 8000 rpm. Discard flow–through and repeat with any remaining sample. (**Hazardous waste note:** The flow-through contains AP3/E buffer with guanidine hydrochloride that is hazardous. Please, discard this fraction according to your lab hazardous waste regulations).
10. Place DNeasy columns into new 2ml collection tubes.
11. Add 500 µ1of Buffer AW and centrifuge for 1 min at 8000 rpm. Discard flow-through.
12. Add 500 µ1of Buffer AW and centrifuge for 2 min at 14000 rpm. Discard flow-through. Make sure membrane is dry, if not return to centrifuge for an addition 30-60 sec. Discard flow- through and keep the column.
13. Place the column in a new 1.5ml tube (you may have to cut off the tube cap to close your centrifuge). Pipet 100µl of pre-heated (65ºC) AE Buffer directly to the membrane at the bottom of the column and take care not to punch through the membrane with the pipet tip. Incubate for 5 min at room temperature. Spin for 1 min at 6,000 x g (8,000 rpm). Discard the column and **keep the flow-through (this is your DNA).** You may want to do a second elution with 200µl of water.
14. Pipette the flow-through (total plant DNA extract) to a new, properly labeled 1.5ml tube if needed and store it at -20ºC.

Note: this protocol can also be used for excised twig phloem. Select phloem tissue from symptomatic twigs of current year growth. Perform test as above, noting weight measurements.

DNA Extraction of Psyllids for Citrus Greening, FDACS-DPI
(Qiagen's DNeasy Tissue Kit, Insect Protocol B with Adaptations)

Before starting extraction, turn on 70°C water bath and float several 1.5ml tubes of molecular biology grade H_20 for eluting DNA.

1. Fill sterile 15ml conical centrifuge tubes with 5 ml of 1X PBS. Place up to 50mg of psyllids into conical centrifuge tubes to equilibrate. Equilibrate tubes at 4°C on a shaker for at least 1 hour, or overnight .

2. Pull out psyllids with tweezers and place in a 1.5 ml tube containing 180µl 1X PBS. Homogenize sample with a sterile Kontes pellet pestle.

3. Add 20µl of Proteinase K and 200 µl of Buffer AL to sample, mix thoroughly by vortexing, incubate for 10 min at 70°C. Invert 2-3 times during incubation to mix.

4. Add 200 µl 100% ethanol and mix thoroughly by inverting tubes.

5. Pipette mixture from step 4, including any precipitate into the DNeasy spin column sitting in a 2ml collection tube. Centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube. Place spin column into a new 2 ml collection tube.

6. Add 500µl of Buffer AW1 and centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube. Place column in a new 2ml collection tube.

7. Add 500µl of Buffer AW2 and centrifuge for 3 min at 14000 rpm to dry the DNeasy membrane. Discard flow-through and collection tube. Place spin column into a new 1.5ml tube.

8. Pipette 50µl of 70°C H_20 onto the DNeasy membrane. Incubate at room temperature for 5 minutes and then centrifuge at 8000 rpm for 1 min to elute DNA.

9. Repeat elution with 200 µl H_20.

Notes: this has been used for combined samples of 4 to 15 individual psyllids, but should work for a single individual, at least with the titer seen in psyllids from Taiwan. If the elution volume is scaled down to ca. 50ul, or if the eluted fraction is precipitated and reconstituted in ca. 20 ul water or buffer, the DNA concentration can be significantly increased to improve sensitivity.

Citrus Greening Conventional PCR Protocol (Asian)
(for DNA from psyllids and leaf material)

For *Ca.* L. asiaticus (Las)
Primers OI1 and OI2c (r16s primers, Jagoueix *et al*. 1996)
(purchased from Invitrogen)

OI1 : 5'-GCG CGT ATG CAA TAC GAG CGG CA-3'
OI2c: 5'-GCC TCG CGA CTT CGC AAC CCA T-3'

PCR Mix (Invitrogen Platinum® Taq DNA Polymerase with 10X and $MgCl_2$)

Chemicals	1 Reaction (µL)	5 Reactions (µL)	Final Concentration
Mol. Grade Water	16.05	80.25	N/A
10X PCR buffer	2.5	12.5	1x
Primers (2µM each)	2.5	12.5	200nM each
MgCl2 (50mM)	1.25	6.25	2.5mM
dNTPs (10mM each)	0.5	2.5	200µM each
Platinum Taq (5U/µ)	0.2	1	1 Unit
Subtotal	23	115	N/A
DNA	2	N/A	N/A
Total	25	25	N/A

Prepare a master mix with the H_20, buffer, dNTPs, primers, and polymerase. Dispense 23 µl into each tube. Add 2 µl of DNA to each tube. Pulse spin tubes, centrifuge, place tubes in thermocycler, and run program "Greening" on the thermocycler.

<u>PCR program Greening</u>

Denaturation: 94°C for 2 min. - 1 cycle

Amplification: 94°C for 30 sec.
62°C for 30 sec. }- 35 cycles
72°C for 60 sec.

Extension: 72°C for 10 min.-1 cycle

Hold: 4°C

Run DNA on a 1.5% agarose gel at 90V for 45 minutes. Use 8 µl of PCR product for electrophoresis. Stain gel in ethidium bromide (EtBr) and visualize product under UV light. Target band is approximately 1200 bp.
==

Citrus Greening Conventional PCR Protocol (Asian and African)

(for DNA from psyllids and leaf material)

To detect both *Ca.* L. asiaticus (Las) and *Ca.* L. africanus (Laf), use the following:
Primers A2 and J5 (rpl (β) operon, Villechanoux *et al*. 1993, Jagoueix *et al*. 1994 and 2000) (purchased from IDT, Inc.)

A2: 5'-TAT AAA GGT TGA CCT TTC GAG TTT-3'
J5 : 5'-ACA AAA GCA GAA ATA GCA CGA ACA A-3'

PCR Mix (Invitrogen Platinum® Taq DNA Polymerase with 10X and $MgCl_2$)

Chemicals	1 Reaction (µL)	5 Reactions (µL)	Final Concentration
Mol. Grade Water	16.05	80.25	N/A
10X PCR buffer	2.5	12.5	1x
Primers (2µM each)	2.5	12.5	200nM each
MgCl2 (50mM)	1.25	6.25	2.5mM
dNTPs (10mM each)	0.5	2.5	200µM each
Platinum Taq (5U/µ)	0.2	1	1 Unit
Subtotal	23	115	N/A
DNA	2	N/A	N/A
Total	25	25	N/A

Prepare a master mix with the H_20, buffer, dNTPs, primers, and polymerase. Dispense 23 µl into each tube. Add 2 µl of DNA to each tube. Pulse spin tubes, centrifuge, place tubes in thermocycler, and run program "Greening" on the thermocycler.

PCR program Greening

Denaturation: 94°C for 2 min. - 1 cycle

Amplification:
94°C for 30 sec.
62°C for 30 sec. }- 35 cycles
72°C for 60 sec.

Extension: 72°C for 10 min.-1 cycle

Hold: 4°C

Run DNA on a 1.5% agarose gel at 90V for 45 minutes. Use 8 µl of PCR product for electrophoresis. Stain gel in ethidium bromide (EtBr) and visualize product under UV light. Target band is approximately 700 bp for both Las and Laf.

==

Citrus Greening Real-Time PCR Protocol (Asian)

The primer-probe combination (HLBaspr) specific *to Candidatus* Libenbacter asiaticus:
HLBas: 5' -TCG AGC GCG TAT GCA ATA CG-3'
HLBp: 5' -56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ 1/ -3'
HLBr: 5' -GCG TTA TCC CGT AGA AAA AGG TAG -3'

The positive internal control primer-probe combination (COXfpr):
COXf. 5' -GTA TGC CAC GTC GCA TTC CAG A -3'
COXp: 5' -/5TET/ATC CAG ATG CTT ACG CTG G/3BHQ 2/ -3'
COXr: 5' -GCC AAA ACT GCT AAG GGC ATT C -3'

2. PCR mix (using Platinum Taq DNA Polymerase from Invitrogen)

Chemicals	1 reaction	5 reactions	Final concentration
Water	4.7 µl	23.5 µl	N/A
$MgCl^2$ (50mM)	3.0 µl	15 µl	6.0 mM
HLBasr (2µM each)	3.0 µl	15 µl	240 nM
HLBp (1µM)	3.0 µl	15 µl	120 nM
COXfr (2µM each)	3.0 µl	15 µl	240 nM
COXp (1µM)	3.0 µl	15 µl	120 nM
10x PCR buffer	2.5 µl	12.5 µl	1x
dNTPs (10mM each)	0.6 µl	2.5 µl	240 µM each
Taq Platinum (5U/µl)	0.2 µl	1 µl	1 Unit
Subtotal	23 µl	115 µl	N/A
DNA extract	2 µl	N/A	N/A
Total	25.0µl	N/A	N/A

3. Amplification program (with a SmartCyc1er II -Cepheid, Sunnyvale, CA) using Dye Set FTTC25:
Stage I: hold at 95°C for 20 s with optics off;
Stage II: 40 cycles
95°C for 1s with optics off
58° for 40 s with optics on
==

Protocol: Asian Citrus Psyllid

If you have questions about Asian citrus psyllid identification, you may ship specimens to your NPDN diagnostic laboratory or contact your local cooperative extension office for information specific to the status of Asian citrus psyllid and citrus greening in your state. Note: Submitted Asian citrus psyllid samples generally are not being tested for citrus greening. For submission of suspect citrus greening samples, see protocols for collecting high-risk plant material.

1-Submitter Shipping:

1. Suspect Asian citrus psyllid samples should be placed in a vial of 100% ethanol *. **Do not mail live suspect Asian citrus samples to your diagnostic clinic.** When shipping alcohol vials, ensure that they are 1) packaged well in a standard mailing tube or strong crush-proof container 2) the seal on the vial is secure and is not likely to be disturbed during shipping and 3) host plant information is also included with each sample submission.

2. Collect multiple, representative samples or life stages. Adults or older nymphs will be easier to identify. Due to their small size, an aspirator is useful in collecting psyllids. Psyllids can be collected by 1) shaking plant material 2) placing a light colored sheet below the host and then 3) aspirating the psyllids.

3. Digital diagnosis may also be useful for screening for the presence of Asian citrus psyllid. Digital diagnosis will not assist you in determining whether or not Asian citrus psyllid is infested with citrus greening bacteria.

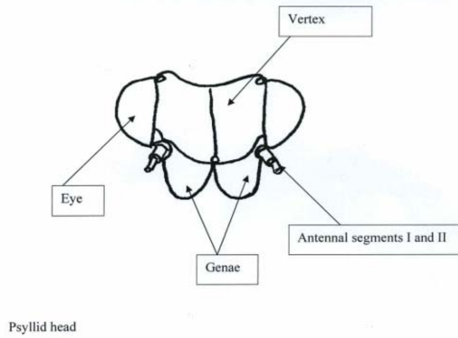
* The US Postal Service considers alcohol a Hazard Class 3: Flammable and Combustible Liquids. To ship through the USPS, it can only be by Surface Mail (no Domestic Airmail, no International). According the USPS regulations, pure ethanol (100%), with a flashpoint of 13-14 degrees C, can be shipped under the following standard:

1. DMM 601.10.13.2.a: "The flashpoint is above 20°F (-7°C) but no more than 73°F (23°C); the liquid is in a metal primary receptacle not exceeding 1 quart, or in another type of primary receptacle not exceeding 1 pint, per mailpiece; enough cushioning surrounds the primary receptacle to absorb all potential leakage; the cushioning and primary receptacle are packed within a securely sealed secondary container that is placed within a strong outer shipping container; and each mailpiece is plainly and durably marked on the address side with "Surface Only" or "Surface Mail Only" and "ORM-D" immediately following or below the proper shipping name."
2. UPS's "SMALL PACKAGE INTERNATIONAL DANGEROUS GOODS ACCEPTED BY UPS -- ONLY FOR APPROVED CUSTOMERS AND ROUTES" found at http://www.ups.com/content/us/en/resources/prepare/idg/download/accepted.html
3. Propylene glycol or vinegar are also good short term preservatives for those concerned with shipping ethanol. If you are requesting DNA extraction from psyllids, consult with your NPDN diagnostic lab prior to shipping in alternative media.

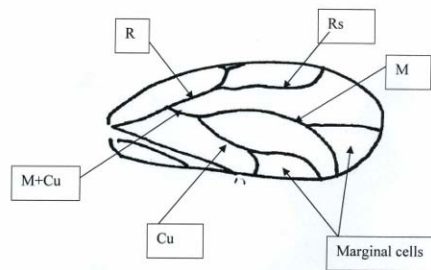
Identification of the Asian Citrus Psyllid

General Identification Overview
The family Psyllidae are generally referred to as the jumping plant lice because adults will readily hop or jump when disturbed. The hind legs of this group are modified for the purpose of jumping. The adult body has a brown, mottled color and is 3 to 4 mm in length. A white, waxy secretion covers the adult insects when living. The head is light brown and wings also have a brown, mottled appearance. Brown bands are evident on the wings, and the antennal tip is black. Nymphs range in size from 0.24 to 1.7 mm, and typically have a yellow to orange color.

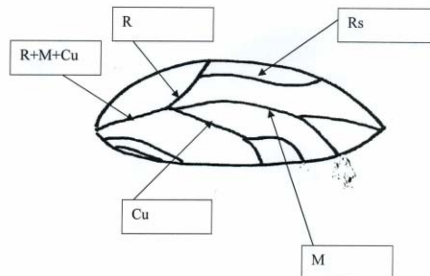
Key Identification Characteristics (modified from information provided by Dr. Susan Halbert at the SPDN 2004 'Homoptera' Workshop)
Slide mounting of genitalia is often necessary for identification confirmation of psyllid species, but the wings, tarsal and tibial spines, the vertex, and genal process can also be useful for identification. Some species of psyllids may actually have enlarged genae that serve a sensory function. Please see the following illustrations.



Psyllid head



Psylla wing



Trioza sp. wing

Some of the key characters of *Diaphorina* (only one species, Asian citrus psyllid in Florida) adult identification include:

1) Vertex is in the same plane with thorax
2) Genae flattened on top, and contiguous with the vertex
3) Wings with brown border and sparse in the middle
4) Genal cones longer than wide

See photos of the adult Asian citrus psyllid:

Photographs by Dr. Susan Halbert, FDACS-DPI

Southern Plant Diagnostic Network (SPDN) Psyllid Identification

Contact your local county extension service or NPDN laboratory. NPDN laboratory personnel with questions concerning psyllid identification should contact their hub laboratory for assistance.

New state records of the Asian citrus psyllid, *Diaphorina citri*, must be confirmed by the USDA-ARS Systematic Entomology Laboratory (address below). New county records can be confirmed by PPQ Identifiers with identification authority of this species, or some state level entomology systematists.

Location Leader
Systematic Entomology Laboratory
Attn: Communication and Taxonomic Services Unit
Building 005, Room 137, BARC-West
10300 Baltimore Avenue
Beltsville, MD 20705
CALL (301) 504-7041

Complete a PPQ form 391 for psyllid samples to be identified at SEL or by PPQ identifiers. Notify Joel Floyd at ppq.nis.urgents@aphis.usda.gov if samples are being forwarded for confirmation of new state or county records.

Selected References for additional Psyllid Taxonomic Information

Burkhardt, D. 1994. Psylloid pests of temperate and subtropical crop and ornamental plants (Hemiptera: Psylloidea): A review Entomol. (Trends in Agril. Sci.) 2: 173-186.

Crawford, D. L. 1914. A monograph of the jumping plant-lice or Psyllidae of the New World. United States National Museum Bulletin 85. Washington, DC 186pp.

Hodkinson, I. D. 1988. The Nearctic Psylloidea (Insecta: Homoptera): an annotated check list. Journal of Natural History 22: 1179-1243.

Hodkinson, I. D. and I. M. White. 1981. The Neotropical Psylloidea (Homoptera: Insecta): an annotated checklist. Journal of Natural History 15: 491-521.

5-Communication:

If the PCR results in a suspect **positive** ID, follow this communications protocol. If a **negative** result is produced, no further communications are necessary.

a. Notify the appropriate NPDN Regional Center of the suspect sample being shipped to the PPQ MDL. Expect notification from Dr. Palm when the sample arrives.

b. Contact the State Plant Health Director (SPHD) and the State Plant Regulatory Official (SPRO) in the sample state of origin,

State Plant Health Director: ______________________________
Address:____
Address:____
Phone Number:____
Fax Number:____
Email:____

State Plant Regulatory Official: ______________________________
Address:____
Address:____
Phone Number:____
Fax Number:____
Email:____

Fax the SPHD at the PPQ regional office:

1. A copy of the updated form 391 with the preliminary diagnosis and the responsible diagnostician's contact information,
2. a copy of the overnight delivery form used to submit the sample to the regional center,
3. and a copy of the state inspector's sample card information submitted with the sample.

c. Notify your Institution's Environmental and Health Safety Official.

EHS Official:____
Address:____
Address:____
Phone Number:____
Fax Number:____
Email: ____

d. Alert Dr. Palm's laboratory that a sample is being forwarded to her laboratory. Provide them with sample shipment time, delivery method, tracking number, sample numbers.

6-Confirmation:

a. Dr. Palm will send results to the PPQ National Identification Services at this e-mail address: ppq.nis.urgents@aphis.usda.gov. Usually Joel Floyd, the PPQ Domestic Diagnostics Coordinator, will in turn notify the Emergency and Domestic Program staff

(usually John Bowers) who will notify the SPHD and SPRO as well as the PPQ regions. The diagnostician will be notified by the SPRO.

b. Notify the Regional NPDN Director of confirmed results.

c. If a positive confirmation is made, state and federal regulatory officials will handle any actions dealing with containment and eradication.

d. Notify your Institution's Environmental and Health Safety Official.

7-Sample Destruction:

Plant material and/or supplies used in the examination and isolation of the suspect sample must be destroyed using a biologically monitored autoclave. The autoclave must be set at a minimum of 15 psi, 121 °C for 30 minutes.

All tools and other equipment must be sanitized and/or sterilized before re-use.

University Documentation (Inactivation of Significant Biological Agents) should be completed upon sample destruction.

Autoclaves are required to be tested periodically for their effectiveness. This can be achieved using a biological monitoring product. Information on one such product can be found at: http://cms.3m.com/cms/US/en/2-21/cirFFFQ/view.jhtml.

Appendix 1: Sampling Methods

- **Complete a sample form.**
  Note total number of symptomatic plants in your report by host type Take a picture of the samples before removing it from the plant if possible. Photograph the entire plant, as well as close-ups of the symptomatic plant part. Step back and photograph the nursery block etc. to gain a perspective of where the symptomatic plant is located with respect to other plants in the nursery. A minimum of 3 photographs per symptomatic plant will be typical, unless several plants in a block are symptomatic, in which case discretion is advised.

- **Sanitation**
  Decontaminate all equipment you use to take samples before leaving a nursery. Use a spray bottle containing a dilute (5%) Clorox® solution or 70% or stronger of ethanol over all tools before leaving the site. Spray boots or shoes with solution in spray bottle before leaving each site.

  Follow decontamination procedures before and after taking each sample.
- **Notification** Ensure that transportation and laboratory facilities have been arranged such that samples will be processed and plated within 24 -48 hours of collection. See Supervisor on where to submit samples.

- **Preparing Samples**

- ✓ Samples should be bagged in a moisture-retaining container, such as a polyethylene bag to prevent drying.
- ✓ Decontaminate hands and place sample bag in a second protective bag.
- ✓ Always write out the identifying label remarks on the outside of the bag.
- ✓ Keep the sample cool and out of the sun (have a foam cooler available).
- ✓ After you have double bagged the sample, fill out a pest and disease sample form and attach it to the bag.

- **Labeling and Documenting Samples**
  After care has been exercised to secure a good sample, it is vital to protect and label the sample properly. The label should contain the complete information pertaining to the sample submitted. Use one of the black felt tipped water proof pens provided. The label must include:
  1. Name and Number of the Nursery or address of property
  2. Time and Date of sampling
  3. Surveyor's ID (alpha-numeric characters) or submitter's name and contact information.
  4. Sample ID - This should be the inspection record ID plus a two character alpha-numeric sample code. Example: [GRN][FL][123][35]

  3 prgm code, 2-letter state code, 3-numeric site code (Nursery) and 2 alpha-numeric sample code.

Appendix 2: Specific Equipment and Reagent Information for DNA Extractions

DNA Extraction Kits:

- DNeasy Plant Mini Kit (Qiagen Cat # 69104)

Reagents and Equipment to Be Supplied by User

- Equipment for disrupting plant tissue. Such as a Bead Beater (see below)
- Ethanol (96–100%)
- Liquid nitrogen

For the DNeasy Plant Mini Kit:

- Microcentrifuge tubes (1.5 ml)
- Microcentrifuge with rotor for 2 ml tubes
- Screw-cap plastic tubes – 2 ml volume (general lab vendors)
- Glass beads 5mm diameter (Fisher Scientific cat. # 11-312C).
- Mini Bead-beater (Cole Parmer, Cat# A-36270-02)

For conventional PCR :

- Thermocycler
- OI1/OI2 or A2/J5primer set
- Molecular grade water
- Invitrogen Platinum® Taq Polymerase (cat # 10966)- includes 10X and $MgCl_2$
- Sigma 10mM dNTP Mix (cat # D 7295)
- Microcentrifuge tubes (1.5 ml and PCR)
- Microcentrifuge with rotor for 2 ml tubes and PCR tubes

For Real-Time PCR :

- Cepheid SmartCycler®
- HLBas/HLBp/HLBr and COXf/COXp/COXr primer-probe sets
- Molecular grade water
- Invitrogen Platinum® Taq Polymerase (cat # 10966)- includes 10X and $MgCl_2$
- Sigma 10mM dNTP Mix (cat # D 7295)
- Microcentrifuge tubes (1.5 ml)
- Cepheid SmartCycler® reaction tubes (cat # 900-0003-pk or 900-0022-cs)

Vendors:

Cole Parmer (800-323-4340) www.coleparmer.com (search for Mini Bead Beater, then select the Mini Bead Beater $847.00)

Cepheid (888-838-3222) www.cepheid.com for SmartCyclers® and tubes

Qiagen (800-426-8157) www1.qiagen.com/products (select Genomic DNA Stabilization and Purification, then select DNA purification from Animal and Plant Samples, be sure to select the MINI kit)

Invitrogen (800-955-6288) www.invitrogen.com

Integrated DNA Technologies (IDT) (800-328-2661) www.idtdna.com for primers and probes

Sigma-Aldrich (800-325-3010) www.sigmaaldrich.com

Fisher Scientific (800-766-7000) www.fishersci.com

Appendix 3: Useful References

Jagoueix, S., Bove, J.M., and Garnier, M. 1996. PCR detection of the two Liberobacter species associated with greening disease of citrus. Molecular and Cellular Probes 10, 43-50.
Chung, K.R. and Brlansky, R. H. 2005. Citrus diseases exotic to Florida: Huanglongbing (Citrus Greening). University of Florida, EDIS PP-210.

De Graca, J.V. 1991. Citrus greening disease. Annu. Rev. Phytopathol. 29:109-136.

Texiera, D.C., Ayres, J., Kitajima, E.W., Tanaka, F.A.O., Danet, L. et al. 2005. First report of a Huanglongbing-like disease of citrus in Sao Paulo State, Brazil, and association of a new Liberibacter species, "*Candidatus* Liberibacter americanus",with the disease. Plant Disease 89:107.

OEPP/EPPO (1988) Data sheets on quarantine organisms No. 151, Citrus greening bacterium and its vectors *Diaphorina citri* & *Trioza erytreae*. *Bulletin OEPP/EPPO Bulletin* **18**, 497-507.

Halbert, S.E. and Manjunath, K.L. 2004. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. Florida Entomologist. 87(3):330-353.

Li, W., Teixeira, D., Hartung, J.S., Levy, L. 2006. Detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing by multiplex real-time pcr. Journal of Microbiological Methods. 66:104-115.

Appendix 4: DNA Extraction Worksheet for DNeasy Plant Mini Kit

DNA EXTRACTION WORKSHEET
DATE:______________________________
Qiagen DNeasy Mini Kit Lot #__________
Technician: _________________________

Supplies:
- Qaigen Kit
- Autoclaved 1.5ml tubes
- Autoclaved glass beads
- Liquid Nitrogen

Sample #	Flow Through Volume (µl)	x 1.5	AP3/E buffer Volume (µl)	√
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		
		1.5		

****It is recommended that you only extract DNA from 12 samples at a time.**
Getting Started:

DNA Extraction* (Modified Protocols of DNeasy Plant Mini Kit - Qiagen, Valencia, CA) Diseased plant samples infected with HLB from Florida and healthy plants samples of sweet orange from the greenhouse of NPGBL in Beltsville are used for total DNA extraction.

____ If using a new kit, add the appropriate amount of 95-100% EtOH to both AW and AP3/E buffer.
____ Turn on the water bath and set @ 65ºC.
____ Pre warm AE buffer in 65ºC water bath.
____ Check AP1 Buffer, it may precipitate out of solution. If so, warm in 65ºC water bath.

Tissue Preparation (should be conducted in a biological safety cabinet)

DNA Extraction (should be conducted in a clean room):

Arrange all the tubes in a rack:

- First row – the QIAshredder columns (lilac colored columns supplied)
- Second row – 1.5 ml tubes

- Third row – DNeasy columns (white columns supplied)
- Forth row – 1.5ml tubes with lids cut off (if necessary).
- Fifth row – 1.5 ml tubes for the DNA extracts clearly labeled with the sample ID and date of extraction.
-

1. Select four twigs of the current year with HLB or HLB-like symptoms collected from the upper canopy o f a suspect tree.

2. Take off the third through the fifth leaves from the top of each twig on a new weighing paper.

3. Get the midribs with petioles from 12 selected leaves using a razor blade and cut them into pieces of about 5 mm in length in a Petri dish.

4. Put 200 mg of small pieces of midribs and petioles into a BIOIOI matrix tube; do triplicate samples for each tree. The rest of the midrib pieces can be stored at -200C for use in the future.

Label all tube caps and the bottom of the lilac column with sample numbers.

---

5. Add 800 ul of buffer API and 8 µl of RNase A stock solution (l00 mg/ml) into a sample tube. If using the Beadbeater instead of BIOIOI matrix tubes, add 600ul of API and 6ul of RNase.

6. Homogenize plant tissues at a speed 6.0 for 40 second with a FastPrep FPI20 instrument using 6 mm cylindrical ceramic sphere (Qbiogene, Carlsbad, CA).

7. Incubate cellular lysate at 65°C for 10 min. with agitation at 300 rpm

8. If using the BIOIOI matrix tubes, add 260 ul of Buffer AP2 to the lysate, vortex briefly and incubate on ice for 5 min. If using the Beadbeater, add 195 ul of buffer.
to the lysate, vortex briefly and incubate on ice for 5 min.

9. Centrifuge at 20,000 x g (14,000 rpm) for 10 min.

10. Transfer the lysate to a QIAshredder Mini Spin Column (lilac) in a 2 ml collection tube and centrifuge for 2 min at 20,000 x g, then, discard the column (typically about 450 ul of lysate can be recovered)

11. Add 1.5 volumes of Buffer AP3/E (675 ul) to the lysate and mix by pipetting.

12. Apply 650 µ l of the mixture including any precipitate to the DNeasy Mini Spin Column sitting in a 2 ml collection tube. Centrifuge at 6000 x g (8000rpm) for 1 min.

13. Repeat Step 12 with the remaining mixture. Discard flow-through and collection tube .

14. Place the column in a new 2 ml collection tube. Add 500 µl of Buffer AW to the column and centrifuge at 8000 rpm for 1 min. Discard flow-through and centrifuge for 2 min at 20,000 x g (14, 000 rpm) to dry the membrane.

15. Record the volume for each sample in the chart in Appendix 4, HLB SOP page 29.

16. Transfer the column to a 1.5 ml microcentrifuge tube and add 100 µ l of Buffer AE onto the column membrane. Incubate for 5 min at room temperature and then centrifuge for 1 min at 6000 x g (8000 rpm) to collect DNA elution. Do the second DNA elution with 100 µl of Buffer AE

17. Extracts of total genomic DNA can be stored at 4° C for immediate use or at -20ºC for use in the future.

* *Sample package opening, sampling and weighing must be conducted in a hood in a BSL2 laboratory. To avoid cross contamination, use a new razorblade, new gloves, new paper towels and, new weighing paper.*

____

The following samples are used in both conventional and real-time PCR reactions:
pl(+) (total DNA extracted from the field-grown plant infected by HLB in Florida), pl(-) (total DNA extracted from the healthy plant in greenhouse in Beltsville), ctl(+) (positive control of total DNA from field-grown plants), and ctl(-) (molecular water).

Validated conventional PCR protocol

1. Primers
Bove's (1996) *16S rDNA-based primer (see reference 1):*
For *Ca.* L. asiaticus (Las) OIl:
5'-GCG CGT ATG CAA TAC GAG CGG CA -3'
OI2C; 5' -GCC TCG CGA CIT CGC AAC CCA T -3'

Bove's (1999) *(β-operon-based primers (see reference 2):*
For both Las and Laf:
A2: 5' -TAT AAA GGT TGA CCT TTC GAG TTT -3'
J5; 5'-ACA AAAGCAGAAATAGCACGA ACAA-3'

2. PCR mix (using Platinum Taq DNA Polymerase from Invitrogen)

Chemicals	1 reaction	5 reactions	Final concentration
Water	16.05 µl	80.25 µl	N/A
lOx PCR buffer	2.5 µl	12.5 µl	Ix
Primers (2 µM each)	2.5 µl	12.5 µl	200nM each
$MgCl^2$(50mM)	1.25 µl	6.25 µl	2.5 mM
dNTPs (10 mM each)	0.5 µl	2.5 µl	200 µM each
Taq Platinum (5U/µl)	0.2 µl	1.0 µl	1 Unit
Subtotal	23 µl	115 µl	N/A
DNA extract	2 µl	N/A	N/A
Total	25.0 µl	125 µl	N/A

3. Amplification program (with MJ Research PTC-200)
94°C for 2 min
35 cycles:
94°C for 30 s
62°C for 30 s for both OIl/OI2c and A2/J5
72ºC for 1 min
72ºC for 10 min

4. Electrophoresis
8.0 µl of PCR product is run on 1.0 agarose at 100V for 1 h, then stain with ethidium bromide.

Real-time PCR Protocol

1. TaqMan primers and probes (Wenbin Li et al., 2006)

The primer-probe combination (HLBaspr) specific *to Candidatus* Liberibacter asiaticus:

HLBas: 5' -TCG AGC GCG TAT GCA ATA CG-3'

HLBp: 5' -56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ 1/ -3'

HLBr: 5' -GCG TTA TCC CGT AGA AAA AGG TAG -3'

The positive internal control primer-probe combination (COXfpr):

COXf. 5' -GTA TGC CAC GTC GCA TTC CAG A -3'

COXp: 5' -/5TET/ATC CAG ATG CTT ACG CTG G/3BHQ 2/ -3'

COXr: 5' -GCC AAA ACT GCT AAG GGC ATT C -3'

2. PCR mix (using Platinum Taq DNA Polymerase from Invitrogen)

Chemicals	1 reaction	5 reactions	Final concentration
Water	4.7 µl	23.5 µl	N/A
$MgCl^2$ (50mM)	3.0 µl	15 µl	6.0 mM
HLBasr (2µM each)	3.0 µl	15 µl	240 nM
HLBp (1µM)	3.0 µl	15 µl	120 nM
COXfr (2µM each)	3.0 µl	15 µl	240 nM
COXp (1µM)	3.0 µl	15 µl	120 nM
10x PCR buffer	2.5 µl	12.5 µl	1x
dNTPs (10mM each)	0.6 µl	3.0 µl	240 µM each
Taq Platinum (5U/µl)	0.2 µl	1 µl	1 Unit
Subtotal	23 µl	115 µl	N/A
DNA extract	2 µl	N/A	N/A
Total	25.0µl	N/A	N/A

3. Amplification program (with a SmartCyc1er II -Cepheid, Sunnyvale, CA) using Dye Set FTTC25:

Stage I: hold at 95°C for 20 s with optics off;

Stage II: 40 cycles

95°C for 1s with optics off

58° for 40 s with optics on

Shipping:

____ Notify Mary Palm's Lab of pending shipment
Record date __________,
time ________________,
and type of notification _______________________________.
____ Create or copy PPQ Form 391 for each sample and include with shipment.
____ Ship to mary Palm's Lab, Attn: Palm, USDA APHIS PPQ CPHST, BARC-East, Bldg-580, 9901 Powder Mill Rd, Beltsville, MD 20705.
Record date __________,
time ________________,
via _________________,
and tracking number____________________.

Appendix 5: Documentation and Specimen Submission Forms

University Documentation

Inactivation of Significant Biological Agents

Name of Significant Biological Agent:____

Accession number and description of diagnostic case:_______________________________

____

____

Date(s) agent was isolated: Amount of agent on site prior to inactivation:

______________________ ____________________________________

Significant agent was:

☐ Inactivated on site

Date:____

Method and description of inactivation:______________________________________

____

If using an autoclave, provide location and cycle conditions (e.g., temperature, pressure, time):_____

____

Witness to the inactivation:

Print name:_______________________Signature:_________________________

☐ Other (provide detailed explanation):____

____

____

I certify that all biological agents isolated by this facility have been inactivated or transferred to a registered facility pursuant to 7 CFR 331, and that all information on this form is true and correct to the best of my knowledge.

Print name:______________________________Signature:_________________________

Date:___________________

Received by Environmental Health and Safety:

Print name:______________________________Signature:_________________________

Date:___________________

University Documentation

Transfer of Significant Biological Agents

Name of Significant Biological Agent:____

Accession number and description of diagnostic case:____

____

____

Date(s) agent was isolated: Amount of agent on site prior to transfer:

______________________________ ____

Significant agent was:

- ☐ Transferred to a registered entity (give name, date, and USDA/APHIS confirmation number):________

____

____

- ☐ All related material was transferred
- ☐ All plant material was transferred
- ☐ A portion of the plant material was transferred
- ☐ All cultures were transferred
- ☐ A portion of the cultures were transferred
- ☐ Other (provide detailed explanation):____

____

____

I certify that all biological agents isolated by this facility have been inactivated or transferred to a registered facility pursuant to 7 CFR 331, and that all information on this form is true and correct to the best of my knowledge.

Print name:____Signature:__________________________________

Date:__________________________

Received by Environmental Health and Safety:

Print name:____Signature:__________________________________

Date:____________________________

Specimen Submission Form that must accompany specimens submitted to the USDA/APHIS National Mycologist.

This report is authorized by law (7 U.S.C. 147a). While you are not required to respond your cooperation is needed to make an accurate record of plant pest conditions.

FORM APPROVED
OMB NO. 0579-0010

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTOIN SERVICE

SPECIMENS FOR DETERMINATION

Instructions: Type or print information requested. Press hard and print legibly when handwritten. Item 1 assign number for each collection beginning with year, followed by collector's initials and collector's number. Example (collector, John J. Dingle); 83-JJD-001. Pest Data Section - Complete Items 14, 15 and 16 or 19 or 20 and 21 as applicable. Complete Items 17 and 18 if a trap was used.

FOR IIBIII USE
LOT NO.
PRIORITY

1. COLLECTION NUMBER

2. DATE — MO | DA | YR

3. SUBMITTING AGENCY
☐ State Cooperator ☐ PPQ ☐ Other ....

4. NAME OF SENDER

5. TYPE OF PROPERTY *(Farm, Feedmill, Nursery, etc.)*

6. ADDRESS OF SENDER

ZIP

INTERCEPTION SITE

7. NAME AND ADDRESS OF PROPERTY OR OWNER

COUNTRY/
COUNTY

8. REASON FOR IDENTIFICATION *("X" ALL Applicable Items)*

A. ☐ Biological Control (Target Pest Name)	E. ☐ Livestock, Domestic Animal Pest
B. ☐ Damaging Crops/Plants	H. ☐ Possible Immigrant (Explain in remarks)
C. ☐ Suspected Pest of Regulatory Concern (Explain in remarks)	J. ☐ Survey (Explain in remarks)
D. ☐ Stored Product Pest	L. ☐ Other (Explain in remarks)

9. IF PROMPT OR URGENT IDENTIFICATION IS REQUESTED, PLEASE PROVIDE A BRIEF EXPLANATION UNDER "REMARKS".

10. HOST INFORMATION
NAME OF HOST *(Scientific name when possible)*

11. QUANTITY OF HOST
NUMBER OF ACRES/PLANTS
PLANTS AFFECTED *(Insert figure & indicate number or percent)* ☐ Number ☐ Percent

12. PLANT DISTRIBUTION
☐ LIMITED
☐ SCATTERED
☐ WIDESPREAD

13. PLANT PARTS AFFECTED

☐ Leaves, Upper Surface	☐ Trunk/Bark	☐ Bulbs, Tubers, Corms	☐ Seeds
☐ Leaves, Lower Surface	☐ Branches	☐ Buds	
☐ Petiole	☐ Growing Tips	☐ Flowers	
☐ Stem	☐ Roots	☐ Fruits or Nuts	

14. PEST DISTRIBUTION
☐ FEW
☐ COMMON
☐ ABUNDANT
☐ EXTREME

15. ☐ INSECTS ☐ NEMATODES ☐ MOLLUSKS

NUMBER SUBMITTED	LARVAE	PUPAE	ADULTS	CAST SKINS	EGGS	NYMPHS	JUVS.	CYSTS
ALIVE								
DEAD								

16. SAMPLING METHOD

17. TYPE OF TRAP AND LURE

18. TRAP NUMBER

19. PLANT PATHOLOGY - PLANT SYMPTOMS *("X" one and describe symptoms)*
☐ ISOLATED ☐ GENERAL

20. WEED DENSITY
☐ FEW ☐ SPOTTY ☐ GENERAL

21. WEED GROWTH STAGE
☐ SEEDLING ☐ VEGETATIVE ☐ FLOWERING/FRUITING ☐ MATURE

22. REMARKS

23. TENTATIVE DETERMINATION

24. DETERMINATION AND NOTES *(Not for Field Use)*

FOR IIBIII USE
DATE RECEIVED
NO.
LABEL
SORTED
PREPARED
DATE ACCEPTED
RR

PPQ FORM 391
(JUL 86)

Previous editions are obsolete.

Appendix 6: List of Primers and Probes for HLB

PCR Reaction	Primer ID	bp length	Sequence (5' - 3')	Purification	Re-Hyd. Conc. (μM)	Stock conc. (μM)	Working conc. (μM)	Aliquot amount (μL)
Conventional-Las only	OI1	23	GCG CGT ATG CAA TAC GAG CGG CA	Standard Desalting	100	20	2	100
	OI2C	22	GCC TCG CGA CTT CGC AAC CCA T	Standard Desalting	100	20	2	
Conventional-Laf only	OA1	22	GCG CGT ATT TTA TAC GAG CGG CA	Standard Desalting	100	20	2	100
	OI2C	22	GCC TCG CGA CTT CGC AAC CCA T	Standard Desalting	100	20	2	
Conventional-Las and Laf	A2	24	TAT AAA GGT TGA CCT TTC GAG TTT	Standard Desalting	100	20	2	100
	J5	25	ACA AAA GCA GAA ATA GCA CGA ACA A	Standard Desalting	100	20	2	
Real-Time-Las Multiplex	HLBas	20	TCG AGC GCG TAT GCA ATA CG	Standard Desalting	100	20	2	100
	HLBr	24	GCG TTA TCC CGT AGA AAA AGG TAG	Standard Desalting	100	20	2	
	HLBp	18	/56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ_1/	HPLC	100	10	1	100
	Coxf CG	22	GTA TGC CAC GTC GCA TTC CAG A	Standard Desalting	100	20	2	100
	Coxr CG	22	GCC AAA ACT GCT AAG GGC ATT C	Standard Desalting	100	20	2	
	Coxp CG	18	/5-TET/ATC CAG ATG CTT ACG CTG G/3BHQ_2	Dual HPLC	100	10	1	100
Real-Time Laf Multiplex	HLBaf	23	CGA GCG CGT ATT TTA TAC GAG CG	Standard Desalting	100	20	2	100
	HLBr	24	GCG TTA TCC CGT AGA AAA AGG TAG	Standard Desalting	100	20	2	
	HLBp	18	/56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ_1/	HPLC	100	10	1	100

	Coxf CG	22	GTA TGC CAC GTC GCA TTC CAG A	Standard Desalting	100	20	2	100
	Coxr CG	22	GCC AAA ACT GCT AAG GGC ATT C	Standard Desalting	100	20	2	
	Coxp CG	18	/5-TET/ATC CAG ATG CTT ACG CTG G/3BHQ_2	Dual HPLC	100	10	1	100
Real-Time Lam Multiplex	HLBam	21	GAG CGA GTA CGC AAG TAC TAG	Standard Desalting	100	20	2	100
	HLBr	24	GCG TTA TCC CGT AGA AAA AGG TAG	Standard Desalting	100	20	2	
	HLBp	18	/56-FAM/AGA CGG GTG AGT AAC GCG/3BHQ_1/	HPLC	100	10	1	100
	Coxf CG	22	GTA TGC CAC GTC GCA TTC CAG A	Standard Desalting	100	20	2	100
	Coxr CG	22	GCC AAA ACT GCT AAG GGC ATT C	Standard Desalting	100	20	2	
	Coxp CG	18	/5-TET/ATC CAG ATG CTT ACG CTG G/3BHQ_2	Dual HPLC	100	10	1	100

Updated July 2008

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Li, W., Hartung, J.S., and Levy, L. 2006. Qualitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. J. of Micr. Methods 104-115. DOI: 10.1016/j.mimet.2005.10.18

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