X-disease phytoplasma and Little Cherry Virus 2

Proficiency test Protocols

Proficiency testing is conducted by WSU with cooperating labs in order to help labs maintain the highest quality outputs for growers. **WSU is not a certifying agency and can only provide testing as a service to the cherry industry.**

Steps:

1. WSU Prosser will obtain samples from known negative and known positive trees (10-20 samples).
2. Samples are pooled, homogenized and a subsample tested by WSU Prosser.
3. Subsamples of pooled samples are provided to participating labs which contain tissue to be tested with accompanying excel submission sheet.
4. For consistency both labs will process the same tissue. Tissue type will depend on timing of analysis, harvest timing = fruit stems, after harvest pooled leaf/bark samples.
5. Extraction and analysis conducted using standardized protocols (**Appendix 1-3**).
6. Sample results from participating labs are provided to WSU Prosser Dr Scott Harper in excel spreadsheet format with column 1 sample name, column 2 LChV2 positive/negative (designate as P/N), column 3 LChV2 Ct value, column 4 XDP positive/negative (designate as P/N), column 5 XDP Ct value.
7. Labs will also provide accompanying run data to allow for Dr. Harper to check that appropriate protocols were used.
8. To pass the panel, all negative samples must be correctly identified, and 85% of results of positives must fall within a range of +6 cycles (Ct values).
9. To be considered for a given panel test results must be returned within 10 working days of sample distribution dates.
10. Labs who do not pass said panel are welcome to submit at the next date but re-analysis will not be accepted.

Contacts

Scott Harper  
Assistant Professor, Department of Plant Pathology  
WSU Irrigated Agriculture Research and Extension Center  
(509) 786-9230  
scott.harper@wsu.edu

Tianna DuPont  
Tree Fruit Extension Specialist  
WSU Tree Fruit Research and Extension Center  
(509) 293-8758  
tianna.dupont@wsu.edu
Appendix 1 Extraction Protocols

The recommended material for testing varies by the time of year. Please refer to the table below to remove the best tissue from the sample provided to you for extraction:

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Timing</th>
<th>Best Tissue</th>
<th>Secondary Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>LChV-1 and LChV-2</td>
<td>Bloom</td>
<td>Current year growth/bark</td>
<td>Flower Buds</td>
</tr>
<tr>
<td></td>
<td>Shuckfall</td>
<td>Current year growth/bark</td>
<td>Flower/Fruit Buds</td>
</tr>
<tr>
<td></td>
<td>Prehavest</td>
<td>Fruit Stem (Pedicel)</td>
<td>Leaf Midrib &amp; Petiole</td>
</tr>
<tr>
<td></td>
<td>Harvest</td>
<td>Fruit Stem (Pedicel)</td>
<td>Leaf Midrib &amp; Petiole</td>
</tr>
<tr>
<td></td>
<td>Postharvest</td>
<td>Current year growth/bark</td>
<td>Leaf Midrib &amp; Petiole</td>
</tr>
<tr>
<td></td>
<td>Predomancy</td>
<td>Current year growth/bark</td>
<td>Leaf Midrib &amp; Petiole</td>
</tr>
<tr>
<td></td>
<td>Dormancy</td>
<td>Current year growth/bark</td>
<td>None</td>
</tr>
<tr>
<td>X-disease phytoplasma</td>
<td>Bloom</td>
<td>Not Recommended</td>
<td>Not Recommended</td>
</tr>
<tr>
<td></td>
<td>Shuckfall</td>
<td>Current year growth/bark</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Prehavest</td>
<td>Current year growth/bark</td>
<td>Fruit Stem (Pedicel)</td>
</tr>
<tr>
<td></td>
<td>Harvest</td>
<td>Current year growth/bark</td>
<td>Fruit Stem (Pedicel)</td>
</tr>
<tr>
<td></td>
<td>Postharvest</td>
<td>Current year growth/bark</td>
<td>Leaf Midrib &amp; Petiole</td>
</tr>
<tr>
<td></td>
<td>Predomancy</td>
<td>TBD</td>
<td>TBD</td>
</tr>
<tr>
<td></td>
<td>Dormancy</td>
<td>Not Recommended</td>
<td>Not Recommended</td>
</tr>
</tbody>
</table>
Nucleic Acid Extraction Type: Total RNA

Method Approved for use on:  Cherry (Prunus avium & P. dulcis)
                            Peach & Nectarine (P. persica)
                            Plum (P. domestica)
                            Apple (Malus sp.)
                            Pear (Pyrus sp.)

Reagents Needed:  Qiagen RNeasy Plant Mini Kit (Qiagen Cat. 74904)
                  Polyvinylpyrrolidone-40 (Sigma Cat. PVP40)
                  Sodium Metabisulfite (Sigma Cat. 31448)
                  Antifoam A Concentrate (Sigma Cat. A6582)
                  20% N-Laurylsarcosine Sodium Salt Solution (Sigma Cat. L5125)

Before Beginning the Extraction:

1. Prepare modified RLC buffer (RLC with 2.5% w/v PVP-40, 0.1% v/v sodium metabisulfite, and
   0.2% v/v Antifoam A).
2. Prepare RPE buffer by adding four volumes of 96-100% ethanol to the stock solution.

Assay Protocol:

1. Weigh 400 mg of scraped bark or stem, petiole, and/or leaf midrib tissue into a grinding bag.
2. Homogenize tissue in 2 mL of modified RLC buffer, then transfer 700 µl of the homogenate into
   a 1.5 ml microcentrifuge tube.
3. Add 84 µl of N-laurylsarcosine solution and incubate at 65 °C for 10 minutes.
4. Centrifuge at 16,100 x g for 4 minutes, then transfer the supernatant to a QIAshredder (purple)
   column and proceed with steps 5-10 OR transfer the supernatant to a 2 ml tube and follow the
   Qiacube process.
5. Centrifuge at 16,100 x g for 2 minutes, then transfer the supernatant to a new tube and add 0.5
   volumes of 96-100% ethanol. Mix and transfer a maximum of 700 µl to an RNeasy spin column
   (pink).
6. Centrifuge at 8,000 x g for 1 minute, and discard the flow-through. Add 700 µl of RW1 buffer
   and centrifuge at 8,000 x g for 1 minute. Again, discard the flow-through.
7. Add 500 µl of RPE buffer to the column and centrifuge at 8,000 x g for 1 minute, then discard
   the flow-through.
8. Add another 500 µl of RPE buffer to the column and centrifuge at 8,000 x g for 1 minute, then
   discard the flow-through.
9. Place the column in a new 2 ml collection tube and centrifuge dry for 1 minute at 16,100 x g.
10. Place the column in a clean 1.5 or 2 ml microcentrifuge tube and add 100 µl of RNase-free H2O.
    Incubate at room temperature for 2 minutes, then centrifuge at 8,000 x g for 1 minute. Discard
    the column and store the samples.
Little Cherry Virus 2: Recommended Protocol for the 2021 Season

**Pathogen:** Little cherry virus-2  
**Type:** One-step real-time RT-qPCR  
**Source:** Wright et al. (Unpublished)  
**Last update:** 2020.12.20

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Orientation</th>
<th>Sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>LChV2-Forward 1</td>
<td>+</td>
<td>CGAGTGTACCTAGTGGTCA</td>
</tr>
<tr>
<td>LChV2-Forward 2</td>
<td>+</td>
<td>GTCTAGCATACTAGTGGACA</td>
</tr>
<tr>
<td>LChV2-Reverse</td>
<td>-</td>
<td>CAAACTCGTCTTTGACATAC</td>
</tr>
<tr>
<td>LChV2-Probe</td>
<td>+</td>
<td>6FAM-CCATCGGAGTTCTGTGAATGCCCTTGBHQ1</td>
</tr>
</tbody>
</table>

**Reagent:** QuantaBio qScript™ XLT One-Step RT-qPCR ToughMix® (Cat. 95132-500)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>1x</th>
<th>Master Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x Reaction Mix</td>
<td>N/A</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Forward Primer 1</td>
<td>600 nM</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Forward Primer 2</td>
<td>600 nM</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>600 nM</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>150 nM</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>RNase-free H2O</td>
<td>N/A</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>RNA template</td>
<td>N/A</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td><strong>20</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Thermocycling conditions:**
- 50 °C for 10 minutes
- 95 °C for 1 minute
- 40 Cycles
- 95 °C for 10 seconds
- 59 °C for 45 seconds

**Notes:**
1. This assay has been optimized for thermocycling temperatures and timings, probe and primer concentration, and Mg²⁺ concentration using the QuantaBio qScript XLT one-step RT-qPCR Toughmix, performance with other reagents cannot be predicted.
2. Both forward primers must be included in the assay to detect LChV-2 genotypes known at the time of development.
3. This assay was optimized using white PCR plates, and their use is recommended.
4. Do not use DEPC-treated water, only ultrapure RNAse-free H₂O.
5. Optimization did not identify a Cq-cutoff value for positives, therefore amplification of all three technical replicates of a given sample within the 40-cycle period was considered an LChV-2 positive. No amplification was observed from known LChV-2 negative samples.
6. Optimization samples were extracted using a modified Qiagen RNeasy Plant Mini kit protocol as described in Beaver-Kanuya et al. (2019) *Journal of Virological Methods* 266: 25-29. Use of crude extraction methods, or methods that cause significant plant polysaccharide or secondary metabolite carryover into the total RNA extract are not recommended.
Appendix 3 X-disease phytoplasma Recommended Protocol for the 2021 Season

**Pathogen:** *Candidatus* Phytoplasma pruni

**Type:** Real-time qPCR

**Source:** Kogej et al. (2020) Development and Validation of a New TaqMan Real-Time PCR for Detection of *'Candidatus Phytoplasma pruni.'* Pathogens 9: 642, [https://doi.org/10.3390/pathogens9080642](https://doi.org/10.3390/pathogens9080642)

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Orientation</th>
<th>Sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>sXd-F</td>
<td>+</td>
<td>GGAATCTCCTCGCTCGCTAAC</td>
</tr>
<tr>
<td>sXd-R</td>
<td>-</td>
<td>AATACCGTTTCCTAYCCCTTTAGAAG</td>
</tr>
<tr>
<td>sXd-Probe</td>
<td>+</td>
<td>6FAM-AGTGGGAGTTCCTACCATTTGAG-BHQ1</td>
</tr>
</tbody>
</table>

**Reagent:** QuantaBio PerfeCTa qPCR ToughMix® (Cat. 95112-012)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>1x</th>
<th>Master Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x Reaction Mix</td>
<td>N/A</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>900 nM</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>900 nM</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>250 nM</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>DNAse/RNase-free H₂O</td>
<td>N/A</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>RNA template</td>
<td>N/A</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td><strong>20</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Thermocycling conditions:**

- 95 °C for 2 minutes
- 45 Cycles
- 95 °C for 15 seconds
- 60 °C for 60 seconds

**Notes:**

7. This assay has been tested using the QuantaBio PerfeCTa qPCR ToughMix, performance with other reagents cannot be predicted.
8. This assay was optimized using white PCR plates, and their use is recommended.
9. Do not use DEPC-treated water, only ultrapure DNAse/RNase-free H₂O.
10. As per the published protocol, samples should be considered as positive if consistent Ct values of less than 40 cycles are obtained across technical replicates.
11. Optimization samples were extracted using a Qiagen DNeasy Plant Mini kit, as per the manufacturer’s instructions. Use of crude extraction methods, or methods that cause significant plant polysaccharide or secondary metabolite carryover into the DNA extract are not recommended.