WASHINGTON STATE UNIVERSITY

# X-disease phytoplasma, Little Cherry Virus-1, and Little Cherry Virus-2 Diagnostic Protocols

The following protocols are recommended based on the research of the WSU Harper Lab as of the last update for X-disease phytoplasma (*Candidatus* Phytoplasma pruni), Little cherry virus 1, and Little cherry virus 2. As further research refines protocols these protocols will be updated.

1. **Tissue sampling and recommended extraction protocols**
2. **X-disease phytoplasma assay**
3. **Little cherry virus 1 assay**
4. **Little cherry virus 2 assay**

Dr. Scott Harper
Assistant Professor, Department of Plant Pathology
(509) 786-9230
scott.harper@wsu.edu

# Appendix 1: Tissue sampling and recommended extraction protocols

## 1. Tissue selection

The recommended material for testing varies by the time of year. Please refer to the table below to select the most optimal tissue for extraction.

| Pathogen | Timing | Best Tissue | Secondary Tissue |
|---|---|---|---|
| LChV-1 and LChV-2 | Bloom | Current year growth/bark | Flower Buds |
| | Shuckfall | Current year growth/bark | Flower/Fruit Buds |
| | Prehavest | Fruit Stem (Pedicel) | Leaf Midrib & Petiole |
| | Harvest | Fruit Stem (Pedicel) | Leaf Midrib & Petiole |
| | Postharvest | Current year growth/bark | Leaf Midrib & Petiole |
| | Predomancy | Current year growth/bark | Leaf Midrib & Petiole |
| | Dormancy | Current year growth/bark | None |
| X-disease phytoplasma | Bloom | Last season's ¼-1/2 in diameter wood/bark with cambium | |
| | Shuckfall | Last season's ¼-1/2 in diameter wood/bark with cambium | |
| | Prehavest | Last season's ¼-1/2 in diameter wood/bark with cambium | Fruit Stem (Pedicel) |
| | Harvest | Last season's ¼-1/2 in diameter wood/bark with cambium | Fruit Stem (Pedicel) |
| | Postharvest | Last season's ¼-1/2 in diameter wood/bark with cambium | |
| | Predomancy | Last season's ¼-1/2 in diameter wood/bark with cambium | None |
| | Dormancy | Not Recommended | Not Recommended |

Tissue types were included in 2022 protocols were based on pathogen distribution in heavily infected trees and in-tree distribution differs signficantly in the early stages of infection. More detailed descriptions can be found in: Katsiani et al. (2018) Plant Disease 102: 899-904, Wright et al. (2021) Archives of Virology 166: 1415-1419, and Wright et al. (2022) Phytopathology, https://doi.org/10.1094/PHYTO-11-21-0468-R. New information from low tighter trees has shown that concentration in the leaf mid-rib and new growth can be highly variable.

## 2. Tissue preparation and homogenization

Ensure that the tissues samples are prepared under sterile conditions and in a manner appropriate to the tissue maceration method being used. As all three pathogens are phloem limited, the maceration method selected must be sufficient to disrupt phloem tissue. We recommend that the tissue be frozen and reduced to a fine powder using bead beating or a BioReba Homex-like system and be kept cold throughout extraction. An example video is available at https://youtu.be/1l3qMZ5uEKE.

## 3. Nucleic acid extraction methods

*Prunus* spp. tissue contains PCR-inhibiting compounds that can affect detection of pathogens by PCR, therefore we recommend the use of a column-based method such as the Qiagen DNeasy Plant Mini Kit for extraction of DNA for detection of the X-disease phytoplasma.

Extraction of good-quality total RNA is even more critical for the detection of LChV-1 and LChV-2, as the one-step RT-qPCR assays used can be hindered by the presence of inhibitory compounds. We therefore recommend a modification of the Qiagen RNeasy Plant Mini Kit detailed below.

# Modified Qiagen RNeasy Plant Mini Kit Extraction Protocol for *Prunus* tissue

**Nucleic Acid Extraction Type**: Total RNA

**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 out the appropriate amount of tissue (see table below) for the maceration method selected, and chop with a razor blade into 2 mm or smaller pieces to ensure good disruption.

| Maceration method | Tissue input range |
|---|---|
| Bead beater | 100 mg |
| Mortar & pestle or equivalent | 100-200 mg |
| BioReba Homex-6 or extraction bag & drill press | 200-400 mg |

2. Homogenize macerated tissue in 500 µl of modified RLC buffer per 100 mg of tissue, then transfer up to 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 column.
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 a RNeasy spin column.
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.

# Appendix 2: X-disease phytoplasma assay

**Pathogen:** *Candidatus* Phytoplasma pruni
**Type:** Real-time qPCR
**Source:** Kogej et al. (2020) Pathogens 9: 642, https://doi.org/10.3390/pathogens9080642

| Primer Name | Orientation | Sequence (5'- -3') |
|---|---|---|
| sXd-F | + | GGAATCTCCTCGCTCGCTAAC |
| sXd-R | - | AATACCGTTTCCTAYCCCTTTAGAAG |
| sXd-Probe | + | 6FAM-AGTGGTCGGAGCCTTCATTAGCATTTGG-BHQ1 |

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

| Reagent | Final Concentration | 1x | Master Mix |
|---|---|---|---|
| 2x Reaction Mix | N/A | 10 | _____ |
| sXd-F (10 µM) | 900 nM | 1.8 | _____ |
| sXd-R (10 µM) | 900 nM | 1.8 | _____ |
| sXd-Probe (10 µM) | 250 nM | 0.5 | _____ |
| DNAse/RNAse-free $H_2O$ | N/A | 3.9 | _____ |
| RNA template | N/A | 2 | _____ |
| **Total Volume:** | | **20** | |

**Thermocycling conditions:**

95 °C for 2 minutes

45 Cycles — 95 °C for 15 seconds; 60 °C for 60 seconds

**Notes:**

1. This assay has been tested using the QuantaBio PerfeCTa qPCR ToughMix, performance with other reagents cannot be predicted.
2. This assay was optimized using white PCR plates, and their use is recommended.
3. Do not use DEPC-treated water, only ultrapure DNAse/RNAse-free $H_2O$.
4. 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. Based on serial dilution of a standard of known concentration we recommend a cut-off threshold of 37 cycles. Depending on tissue condition and type, positives can be obtained above that value but may be inconsistent.
5. 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.

# Appendix 3: Little cherry virus-1 assay

**Pathogen:** Little cherry virus-1
**Assay Type:** One-step real-time RT-qPCR
**Assay Source:** Katsiani et al. (2018) Plant Disease 102: 899-904

| Primer Name | Orientation | Sequence (5'- -3') |
|---|---|---|
| LCh-A | + | CCAATGCACAAAGCACATATGA |
| LCh-B | + | CCAATGCATAAAGCTCATATGA |
| LCh-DF | + | CCAATGCACAAAGCATCAATGA |
| LCh-C | - | ACCGCGACGTGGTCCTAATA |
| LCh-D | - | ACCTCGACGTGGTCCCAATA |
| LCh-DR | - | ACCACGACGTGGTCCTAACA |
| LCh-P3 | + | 6FAM-TCGAARGGAGCTCTYCATGTTTCGCA-BHQ1 |

**Controls to be used:**

1. Positive — LChV-1 positive tissue
1. Negative — Virus-free *Prunus* tissue
1. No-Template — Water

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

| Reagent | Concentration | 1x | Master Mix |
|---|---|---|---|
| 2x Reaction Mix | N/A | 10 | _____ |
| LCh-A (10 µM) | 250 nM | 0.5 | _____ |
| LCh-B (10 µM) | 250 nM | 0.5 | _____ |
| LCh-DF (10 µM) | 250 nM | 0.5 | _____ |
| LCh-C (10 µM) | 250 nM | 0.5 | _____ |
| LCh-D (10 µM) | 250 nM | 0.5 | _____ |
| LCh-DR (10 µM) | 250 nM | 0.5 | _____ |
| LCh-P3 (10 µM) | 100 nM | 0.2 | _____ |
| RNAse-free $H_2O$ | N/A | 4.8 | _____ |
| RNA template | N/A | 2 | _____ |
| **Total Volume:** | | **20** | |

**Thermocycling conditions**
50 °C for 10 minutes
95 °C for 1 minute
40x 95 °C for 10 seconds
60 °C for 45 seconds

**Notes:**

1. This assay has been optimized for thermocycling temperatures and timings, probe and primer concentration, and $Mg^{2+}$ concentration using the QuantaBio qScript XLT one-step RT-qPCR Toughmix, performance with other reagents cannot be predicted.
2. All primers and probes must be included in the assay to detect LChV-1 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 $H_2O$.
5. 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 4: Little Cherry Virus 2 assay

**Pathogen:** Little cherry virus-2
**Type:** One-step real-time RT-qPCR
**Source:** Shires et al. (2022) Plant Disease https://doi.org/10.1094/PDIS-08-21-1769-RE

| Primer Name | Orientation | Sequence (5'- -3') |
|---|---|---|
| LChV2-Forward 1 | + | CGAGTGTACCTAGTGGTCA |
| LChV2-Forward 2 | + | GTCTAGCATACCTAGTGGACA |
| LChV2-Reverse | - | CAAACTCGTCTTGTGACATAC |
| LChV2-Probe | + | 6FAM-CCATCGGAGTTCTGTGAATGCCTTG-BHQ1 |

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

| Reagent | Final Concentration | 1x | Master Mix |
|---|---|---|---|
| 2x Reaction Mix | N/A | 10 | |
| LchV-2-Forward 1 | 600 nM | 1.2 | |
| LchV-2-Forward 2 | 600 nM | 1.2 | |
| LchV-2-Reverse | 600 nM | 1.2 | |
| LchV-2-Probe | 150 nM | 0.3 | |
| RNAse-free $H_2O$ | N/A | 4.1 | |
| RNA template | N/A | 2 | |
| **Total Volume:** | | **20** | |

**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 $Mg^{2+}$ 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 $H_2O$.
5. Per Shires at al. (2022) a cut off threshold value of 37 is recommended.
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.