

## **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 (12 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 3 cycles (Ct values).

## Appendix 1 Extraction Protocols



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CPCNW-VDL Request No. \_\_\_\_\_

Performed by: \_\_\_\_\_

Date: \_\_\_\_\_

### 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 H<sub>2</sub>O. 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 Little Cherry Virus 2



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CPCNW-VDL Request No. \_\_\_\_\_

Performed by: \_\_\_\_\_

Date: \_\_\_\_\_

**Pathogen:** *Little cherry virus 2 (Ampelovirus)*

**Assay Type:** Real-Time PCR

**Assay Source:** Harper et al. (unpublished)

Primer Name	Orientation	Sequence (5' - 3')	Binding Site	Fluorophore
LChV2-RT-F	+	GCGGAGGGTCTGGAGACTT	6769-6787	FAM
LChV2-RT-R	-	AGGCCAGGAGACAAACAACATC	6832-6850	
LChV2-RT-P	+	TGAGTCTTTCAAGGACAC	6789-6806	

**Controls to be used:**

1. Positive *Little cherry virus-2* positive tissue
2. Negative Virus-free *Prunus* tissue
3. No-Template Water

**Reagents to be used:** Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit (11732-020)

**PCR Hood Number:** \_\_\_\_\_ **qPCR Machine Number:** \_\_\_\_\_

**2x Master Mix Lot Number:** \_\_\_\_\_ **Date Opened** \_\_\_\_\_

**Enzyme Lot Number:** \_\_\_\_\_ **Date Opened** \_\_\_\_\_

**Number of reactions needed:** \_\_\_\_\_ *Add 1 additional reaction to account for wastage*

Reagent	Concentration	1x	Master Mix	<b>Thermocycling conditions</b> 50 °C for 15 minutes 94 °C for 2 minutes 40x 94 °C for 10 seconds 60 °C for 45 seconds
2x Reaction Mix	N/A	10	_____	
Forward Primer (10 µM)	400 nM	0.8	_____	
Reverse Primer (10 µM)	400 nM	0.8	_____	
Probe (10 µM)	100 nM	0.2	_____	
SSIII/Taq enzyme mix	N/A	0.4	_____	
RNAse-free H <sub>2</sub> O	N/A	5.8	_____	
RNA template	N/A	2	_____	
<b>Total Volume:</b>		<b>20</b>		

Please attach the real-time results spreadsheet to the back of this worksheet, add any comments in the space below, and attach the completed worksheet to the back of the diagnostic request form.

## Appendix 3 X-disease phytoplasma



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CPCNW-VDL Request No. \_\_\_\_\_

Performed by: \_\_\_\_\_

Date: \_\_\_\_\_

**Pathogen:** Phytoplasma

**Assay Type:** Real-Time PCR

**Assay Source:** Ito and Suzuki (2017) PLoS ONE 12(9): e0185427

Primer Name	Orientation	Sequence (5' - 3')	Binding Site	Fluorophore
UPH-F (F)	+	CGTACGAAGTATGAACTTAAAGGA	280281-280299	FAM
D-UPHr2 (R)	-	CGACAACCATGCACCACCTGIIIICTGATAACC	280442-280423	
UPH-Pb (P)	+	TGACGGGACTCCGCACAAGCG	280302-280322	

**Controls to be used:**

1. Positive      Phytoplasma positive tissue
2. Negative      Phytoplasma-free *Vitis* or *Prunus* tissue
3. No-Template      Water

**Reagents to be used:** Applied Biosystems TaqMan Universal PCR Master Mix (4304437)

**PCR Hood Number:** \_\_\_\_\_ **qPCR Machine Number:** \_\_\_\_\_

**2x Master Mix Lot Number:** \_\_\_\_\_ **Date Opened** \_\_\_\_\_

**Number of reactions needed:** \_\_\_\_\_ *Add 1 additional reaction to account for wastage*

Reagent	Concentration	1x	Master Mix	<b>Thermocycling conditions</b> 50 °C for 2 minutes 95 °C for 10 minutes 40x 95 °C for 15 seconds 60 °C for 60 seconds
2x Reaction Mix	N/A	10	_____	
Forward Primer (10 µM)	300 nM	0.6	_____	
Reverse Primer (10 µM)	300 nM	0.6	_____	
Probe (10 µM)	100 nM	0.2	_____	
DNA template	N/A	2	_____	
RNAse-free H <sub>2</sub> O	N/A	6.6	_____	
<b>Total Volume:</b>		<b>20</b>		

Please attach the real-time results spreadsheet to the back of this worksheet, add any comments in the space below, and attach the completed worksheet to the back of the diagnostic request form.