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**[1]DRAFT ANNEX to ISPM 27: ‘Candidatus Liberibacter’ spp. on Citrus spp. (2004-010)**

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<b>[3]</b> This is not an official part of the standard and it will be modified by the IPPC Secretariat after adoption.	
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<b>[31]Main discussion points during development of the diagnostic protocol</b>	<b>[32]</b>
<b>[33]Notes</b>	<b>[34]</b> This is a draft document. <b>[35]</b> 2021-04 Edited

**[36]**

**[37]CONTENTS**

**[38][to be added later]**

**[39]Adoption**

**[40]**This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in [Month 20--]. [to be completed after adoption]

**[41]**The annex is a prescriptive part of ISPM 27 (*Diagnostic protocols for regulated pests*).

## [42]1. Pest information

[43]Huanglongbing (HLB), caused by ‘*Candidatus Liberibacter*’ species and also known as citrus greening, is one of the most destructive and widespread diseases of citrus in Asia, Africa and the Americas, affecting mainly *Citrus* species, cultivars and hybrids<sup>1</sup> and, to a lesser extent, some other hosts within the Rutaceae (EPPO, 2014; CABI, 2021). The ‘*Ca. Liberibacter*’ species associated with the disease are transmitted by the psyllids *Diaphorina citri* (EPPO, 2005), *Trioza erytreae* and *Cacopsylla citrisuga* (Cen *et al.*, 2012); ‘*Candidatus Liberibacter asiaticus*’ was also detected in *Diaphorina communis* identified in Bhutan (Donovan *et al.*, 2012).

[45]Huanglongbing-like symptoms were described for the first time in 1919 in China and then in 1921 in the Philippines (Bové, 2006). However, farmers in southern China had observed yellowing of their citrus trees since the late 1800s and in India the “citrus dieback” syndrome has been reported since the eighteenth century. In South Africa, the first symptoms of HLB were recorded in 1828/29 (Da Graça, 2010). Subsequently, HLB has extended its distribution into many of the major citrus-producing areas of the world.

[46]The causal agents of HLB are fastidious Gram-negative bacteria in the ‘*Ca. Liberibacter*’ genus (Garnier, Danel and Bové, 1984). ‘*Ca. Liberibacter*’ species are restricted to the sieve tubes within the phloem tissues, occur at very low concentrations and are unevenly distributed within the host plant (Jagoueix, Bové and Garnier, 1994). The pathogenic ‘*Ca. Liberibacter*’ species were discovered by electron microscopy in citrus trees with HLB symptoms. Three species of ‘*Ca. Liberibacter*’ have been associated with HLB and are differentiated based on the nucleotide sequence in the 16S ribosomal gene operon (Jagoueix, Bové and Garnier, 1994). The three species are as follows:

- [47]‘*Candidatus Liberibacter asiaticus*’, transmitted by *Diaphorina citri*, is heat tolerant and induces symptoms in warm climates at optimal temperatures in the range 27–32 °C (Jagoueix *et al.*, 1996). It is present in Asia, Africa, Oceania and North and South America (Bové, 2006; da Graça, 2010; CABI, 2021).
- [48]‘*Candidatus Liberibacter africanus*’ is transmitted by *Trioza erytreae*, is heat-sensitive and causes symptoms between 22 °C and 24 °C (Jagoueix, Bové and Garnier, 1994), with no symptoms appearing at 27–30 °C (da Graça, 1991). It is present in Asia (Saudi Arabia and Yemen) and Africa (Ascension, Saint Helena and Tristan da Cunha; Burundi; Cameroon; the Central African Republic; the Comoros; Ethiopia; Kenya; Madagascar; Malawi; Mauritius; Mayotte; Réunion; Rwanda; Somalia; South Africa; Swaziland; the United Republic of Tanzania; and Zimbabwe) (Bové, 2006; da Graça, 2010; CABI, 2021). ‘*Candidatus Liberibacter africanus* subsp. *capensis*’ has been reported in South Africa on an ornamental rutaceous tree, *Calodendrum capense* (Garnier *et al.*, 2000).
- [49]‘*Candidatus Liberibacter americanus*’ was described as a new species when it was first found in 2004 in São Paulo, Brazil (Teixeira *et al.*, 2005a, 2005b, 2005c; Bové, 2006). It is also transmitted by *Diaphorina citri* (Yamamoto *et al.*, 2006). ‘*Ca. L. americanus*’ is less heat tolerant than ‘*Ca. L. asiaticus*’. Lopes *et al.* (2009) found that temperatures above 32 °C negatively affected the multiplication of ‘*Ca. L. americanus*’ in infected plants, whereas ‘*Ca. L. asiaticus*’ was affected only by temperatures above 38 °C. Similarly, Gasparoto *et al.* (2012) found that ‘*Ca. L. americanus*’ did not infect plants maintained at night/day temperature conditions of 27/32 °C, but infection by ‘*Ca. L. asiaticus*’ occurred at all the studied temperatures.

[50]Huanglongbing is a disease limited to *Citrus* and a few other genera of Rutaceae. The disease is present in *C. aurantiifolia* (lime), *C. ×aurantium* (sour orange), *C. limonia* Osbeck (Rangpur lime), *C. limon* L. (lemon), *C. limettioides* (Palestinian sweet lime), *C. japonica* (syn. *Fortunella japonica*) (kumquat), *C. medica* (citrons), *C. paradisi* (grapefruit), *C. paradisi* × *C. reticulata* (tangelo), *C. reticulata* (mandarin), *C. sinensis* (L.) Osbeck (sweet orange) and *Poncirus trifoliata* (trifoliate orange) (da Graça, 1991). The rutaceous trees *Calodendrum capense* (Cape chestnut), *Murraya*

□

[44]<sup>1</sup> *Citrus* species, cultivars and hybrids are named according to the International Plant Names Index: <https://www.ipni.org>

*paniculata* (Garnier *et al.*, 2000, Lopes *et al.*, 2010) and *Atalantia* (syn. *Severinia*) *buxifolia* (Deng *et al.*, 2008) also harbour ‘*Ca. Liberibacter*’ species and support populations of *Trioza erytreae* and *Diaphorina citri* (Garnier *et al.*, 2000), Jagoueix *et al.*, 1996). Other hosts may be viewed at <https://gd.eppo.int/taxon/LIBEAS/hosts>.

[51]The psyllids reported as being the vectors of the HLB agents persist and multiply on other rutaceous plants including *A. buxifolia*, *Atalantia missionis*, *Citrus inodora*, *Citrus ×virgata* Mabb ‘Sydney Hybrid’, *Citropsis gabunensis*, *Citropsis schweinfurthii*, *Clausena anisum-olens*, *Limonia acidissima*, *Naringi crenulata* (Barkley and Beattie, 2008), *Swinglea glutinosa* (Garnier and Bové, 1993), and *Vepris lanceolata* (Gottwald *et al.*, 2007).

[52]To date, psyllids are the only group of insects known to transmit ‘*Ca. Liberibacter*’ spp. (Cen *et al.*, 2012). The bacterium can multiply in the body of the insect vectors (Aubert, 1987 and Jagoueix, Bové and Garnier, 1997). Pelz-Stelinsky *et al.* (2010) reported that transmission of ‘*Ca. L. asiaticus*’ from parent to offspring (transovarial) occurred at a rate of 2–6%.

## [53]2. Taxonomic information

[54]**Name:** ‘*Candidatus Liberibacter africanus*’ Garnier, 2000

[55]**Synonym:** ‘*Candidatus Liberobacter africanus*’ Jagoueix *et al.*, 1994

[56]**Name:** ‘*Candidatus Liberibacter americanus*’ Teixeira *et al.*, 2005

[57]**Name:** ‘*Candidatus Liberibacter asiaticus*’ Garnier, 2000

[58]**Synonym:** ‘*Candidatus Liberobacter asiaticus*’ Jagoueix *et al.*, 1994

[59]**Taxonomic position:** Bacteria, Proteobacteria, Alpha-Proteobacteria, Rhizobiales, Rhizobiaceae

[60]**Disease names:** Huanglongbing (HLB) or citrus greening. The common name “huanglongbing” is currently widely adopted in the scientific literature (CABI, 2021).

[61]In 1994, the International Committee for Systematic Bacteriology recommended that, as proposed by Murray and Schleifer (1994), a ‘*Candidatus*’ designation be used as an interim taxonomic status, to provide a proper allocation of sequence-based potential new taxa at the genus and species level (Murray and Schleifer, 1994; Murray and Stackebrand, 1995). Jagoueix, Bové and Garnier (1994) proposed that this new group in the alpha subdivision of the Proteobacteria should be referred to by the name “liberobacter” (from the Latin *liber* [bark] and *bacter* [bacteria]). Subsequently, two “*Candidatus* species”, *Liberobacter asiaticum* and *Liberobacter africanum*, were recognized based on polymorphism in the 16S rDNA nucleotide sequences. Later, the spelling was corrected to ‘*Liberibacter*’ to conform to the Latin convention of using the connecting vowel “i” rather than “o” (Garnier *et al.*, 2000). Garnier *et al.* (2000) proposed, based on serological differences and phylogenetic analysis, the name ‘*Ca. Liberibacter africanus* subsp. *capensis*’ for an isolate obtained from *Calodendrum capense*. In 2004, a new species was discovered in Brazil that failed to amplify with primers designed for ‘*Ca. Liberibacter asiaticus*’ and ‘*Ca. Liberibacter africanus*’ in PCR and the new strain was named ‘*Ca. L. americanus*’ Teixeira *et al.* (2005c).

## [62]3. Detection

[63]Huanglongbing was diagnosed in the late twentieth century by conventional procedures such as electron microscopic examination and by bioassays on indicator plants. The ‘*Candidatus Liberibacter*’ species associated with HLB have not yet been cultured *in vitro*, but methods based on the polymerase chain reaction (PCR) amplification of sequences from genes, such as the 16S ribosomal (r)RNA gene and the *rplK**AJL-rpoBC* gene cluster, are considered efficient and sensitive for the detection of liberibacters in HLB-infected plant tissue and psyllids.

[64]The use of PCR to detect ‘*Ca. Liberibacter*’ spp. in a vector is a very useful tool for surveillance because it allows detection of the pathogen in the insect before the appearance of the symptoms.

Molecular detection is the method that may detect the bacterium in a single adult or in the third, fourth and fifth instars of the psyllid (Manjunath *et al.*, 2008). Nguyen, Le and Nguyen (2003) showed that HLB-infected psyllids contain a higher titre of the bacterium than HLB-infected plant tissue.

[65] Loop mediated isothermal amplification (LAMP) has been adapted for the sensitive detection of ‘*Ca. L. asiaticus*’ (Okuda *et al.*, 2005, Rigano *et al.*, 2014; Keremane *et al.*, 2015; Choi *et al.*, 2018). Such LAMP-based methods are performed at a constant temperature, can be used on crude DNA extractions, and have shown promise for on-site diagnostics. However, these methods have not yet been well validated for routine diagnosis of ‘*Ca. L. asiaticus*’ and hence are not included in this diagnostic protocol.

[66] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

### [67] 3.1 Symptoms

[68] Inspection is important for detection in symptomatic plants and is a routine method for the surveillance of HLB in areas where the disease is not present. Yellow shoots and blotchy mottle symptoms on leaves are typical symptoms on HLB-infected trees and can be used on site as part of an initial diagnosis. However, symptoms can be confused with nutritional disorders (zinc, iron or manganese deficiencies) or with other diseases (e.g. Australian citrus dieback (‘*Candidatus Phytoplasma*’ sp.), citrus blight, *Citrus tristeza virus*, stubborn disease of citrus ( *Spiroplasma citri*)). ‘*Ca. Liberibacter*’ spp. can also be present in the host plant at a very low concentration and can be unevenly distributed in the host plant, resulting in sparse symptoms that are easy to miss.

[69] Symptoms of HLB develop slowly. Infected trees gradually decline in vigour and yield and remain stunted or eventually die (Figure 1). The disease develops irregularly, so individual trees may show a mixture of normal and diseased sectors (Figure 2 and 3). This mixture within the same tree is a diagnostic characteristic (see <https://iocv.ucr.edu/sites/g/files/rcwecm4696/files/huanglongbing-011.jpg>).

[70] Symptoms first appear as leaf yellowing, followed by mottling and chlorosis in one shoot or sector of the tree. Later, leaf symptoms resemble nutritional deficiencies (zinc, copper or nitrogen) but may vary depending on the bacterial strain. The larger leaves on the base of branches turn yellow along the main and secondary veins and later change to a “blotchy mottle”, with the two halves of the leaf being asymmetrical in terms of the pattern of yellow and green. This is the most characteristic foliar symptom (see <https://iocv.ucr.edu/sites/g/files/rcwecm4696/files/huanglongbing-041.jpg>).

[71] As the discoloration spreads away from the veins, the leaves become pale to light yellow with unevenly distributed dark green patches. Leaves on weak terminal twigs are small, upright and show a variety of chlorotic patterns. Infected fruits have a bitter and salty taste and a reduced Brix ratio, are smaller and of poor quality, often fail to develop normal fruit colour (colour inversion), and can fall prematurely (see <https://iocv.ucr.edu/sites/g/files/rcwecm4696/files/huanglongbing-085.jpg>).

[72] The columella is curved, causing the fruit to be distorted and lopsided (see <https://iocv.ucr.edu/sites/g/files/rcwecm4696/files/huanglongbing-092.jpg>). Seeds in the affected fruit are usually abortive.

[73] Images of HLB symptoms and distinctive characteristics may be viewed at the EPPO Global Database (<https://gd.eppo.int/taxon/LIBEAS/photos> or <https://iocv.ucr.edu/citrus-diseases/huanglongbing>).

### [74] 3.2 Sampling and sample preparation

[75] Huanglongbing is a systemic disease of citrus, and ‘*Ca. L. asiaticus*’ has been detected in bark tissue, leaf midrib, roots, and different floral and fruit parts of infected citrus trees (Tatineni *et al.*, 2008).

### [76]3.2.1 Symptomatic material

[77]An appropriate sample from a symptomatic tree consists of five to ten leaves (NAPPO, 2012). Tissue prints of the petioles or the basal part of the leaves on membranes can also be used, as described by Bertolini *et al.* (2014) and Siverio *et al.* (2017) (see section 3.4.1). The leaf samples are placed in a labelled plastic bag (one bag per tree), stored in a cool box while in the field, and refrigerated as soon as possible. Leaves can be processed at any time up to three weeks after collection if kept in sealed plastic bags or other sealed containers at 4 °C and if no decay has occurred. The midribs of collected leaves are excised and processed for DNA extraction because the leaf midribs are enriched in phloem vessels and as consequence have a higher titre of '*Ca. Liberibacter*' cells. The older leaves and longer infected plants yield a higher titre of *Liberibacter* DNA (Nguyen, Le and Nguyen, 2003). There is also a higher titre of bacterial DNA in sieve tube cells of citrus leaf midribs than in lamina tissue (da Graça, 1991; Wang *et al.*, 2006).

### [78]3.2.2 Asymptomatic material

[79]An appropriate sample from a symptomless tree consists of at least ten mature leaves collected from around the canopy of a tree (EPPO, 2014). For small trees (e.g. in a nursery), three to four leaves per tree are collected. The sampled leaves are placed in a labelled plastic bag (one bag per tree), stored in a cool box while in the field, and refrigerated as soon as possible. Leaves should be kept in sealed plastic bags or sealed containers at 4 °C and processed as soon as possible.

### [80]3.2.3 Psyllids

[81]The preparation of the specimen or specimens consists of placing the adults or nymphs in a labelled vial and then either processing them for DNA extraction immediately or preserving them in 70% ethanol. The insects may also be squashed onto membranes (see section 3.4.2).

### [82]3.3 Biological detection (graft transmission)

[83]Biological indexing is a reliable technique for '*Ca. Liberibacter*' species detection despite the low rate of graft transmission and is suitable as a screening test for use by diagnosticians who have experience with symptom observation. The indicators used commonly are *C. sinensis* (sweet orange) or *C. reticulata* (mandarin) for '*Ca. L. asiaticus*', sweet orange or (*Citrus x tangelo* (Orlando tangelo) for '*Ca. L. africanus*', and *C. sinensis* or *C. reticulata* × *C. sinensis* (Murcott tangor) for '*Ca. L. americanus*' (Lopes and Frare, 2008; NAPPO, 2012). *Catharanthus roseus* (periwinkle) may also be used: in this host, HLB can multiply and is present at a higher titre than in citrus plants after transmission by *Cuscuta campestris* (dodder) (Garnier and Bové, 1983), with the symptoms developing after three months at 25 °C (Bové, 2006; Nguyen, Le and Nguyen, , 2003).

[84]There are several recommendations for selecting plant material for grafting onto indicator plants. According to Lopes *et al.* (2009), the best inoculum is from symptomatic branches (particularly those showing symptoms within the previous 12 months) that are suspected to be infected by any '*Ca. Liberibacter*' spp. The selected branch piece is cut into segments, each 2–4 cm long, and the segments are grafted onto the opposite side of the indicator twig. After inoculation, the graft is protected with polyethylene tape, the grafted indicator twig is protected with a polyethylene bag, and the plants are maintained in a greenhouse. The grafted indicator plants are then inspected regularly (one or twice a month). The first symptoms appear at four or five months after inoculation with yellowing of the apical leaves (similar to manganese or iron deficiencies) and progress to the appearance of blotchy mottled leaves showing a diffuse and asymmetrical light chlorosis after 6 to 12 months (Lopes and Frare, 2008; EPPO, 2014).

[85]Leaf grafting is performed using a 3 × 12 mm section of the midrib part of the leaf, placed into a T-cut in the bark of an indicator seedling (Roistacher, 1991). The grafted plants are kept at 20–25 °C for '*Ca. Liberibacter africanus*' and at 25–32 °C for '*Ca. Liberibacter asiaticus*' (EPPO, 2014). It has been demonstrated that '*Ca. L. asiaticus*' is transmitted more efficiently than '*Ca. L. americanus*' and reaches a higher titre in the infected plant (Hall *et al.*, 2012).



### [86]3.4Molecular detection

[87]Although conventional PCR is relatively sensitive and specific, this test can lead to false negative results when the concentration of the bacterium is too low to detect, for instance in newly infected trees with a low concentration and uneven distribution of the pathogen (Bové, 2006). Consequently, conventional PCR should only be used on plants exhibiting symptoms and is not reliable for the detection of '*Ca. Liberibacter*' spp. in symptomless plants. However, real-time PCR may be useful in programmes for the production of certified citrus nursery trees and in post-entry quarantine. Li *et al.* (2006) and Bertolini *et al.* (2014) have reported that real-time PCR can detect '*Ca. Liberibacter*' spp. in symptomless plants and is more convenient for early detection.

#### [88]3.4.1 Nucleic acid extraction from plant material

[89]Based on experience from several countries, the following methods may be used to extract DNA from plant tissue, although other DNA extraction kits can also give good DNA quality.

[90]**CTAB extraction.** The plant tissue (500 mg midribs) is disrupted either by use of commercially available equipment (a Fastprep (MP Biomedicals) instrument or a Mini-Beadbeater (BioSpec) instrument) or manually by grinding with a mortar and pestle or by crushing the tissue in a plastic bag. Cetyl trimethyl ammonium bromide (CTAB) buffer (3 mL) containing 0.2%  $\beta$ -mercaptoethanol is added and stirred. After this, 2 mL homogenate is transferred to a microtube and incubated, if possible with shaking, for at least 15 min at 65 °C. The resulting extract is centrifugated at 3 000 g for 5 min in a microcentrifuge and 1 mL supernatant is then put in a 2 mL microtube with 1 mL chloroform-isoamyl alcohol solution (24:1), mixed and centrifugated at 14 000 g for 5 min. The aqueous phase is transferred to a new microtube, mixed with 0.6 volume of cold isopropanol and kept at -20 °C for 30 min before centrifugation at 14 000 g for 20 min. The supernatant is discarded and the pellet washed twice with 70% ethanol and resuspended in 100  $\mu$ L sterile distilled water. The resulting extracts can be stored at -20 °C.

[91]**Commercial kits.** After using any of the disruption methods described above in relation to CTAB extraction, DNA extraction is carried out using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

[92]**Plant tissue print.** The plant tissue print method is a rapid, direct method of sample preparation (Bertolini *et al.*, 2008) that can be done under field conditions and has demonstrated its efficiency when combined with the real-time PCR detection method of Bertolini *et al.* (2014). The tissue print method is performed by pressing five to ten fresh, manually detached, citrus leaf petioles onto an area (0.5 cm<sup>2</sup>) of a positively charged nylon or 3MM filter paper membrane (Bertolini *et al.*, 2008). The tissue printed membrane is cut out and inserted into microcentrifuge tubes containing either 100  $\mu$ L distilled water, 0.5% Triton X-100 or glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)). Samples are then incubated at 100 °C for 10 min as described by Bertolini *et al.* (2014), vortexed and placed on ice until use.

#### [93]3.4.2 Nucleic acid extraction from the psyllid vectors

[94]**Manjunath *et al.* (2008).** In this method, the psyllids (up to 50) are air-dried for 10 min, transferred to a 1.5 mL microtube containing 300  $\mu$ L extraction buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 2% Sodium dodecyl sulphate) and 20 units of Proteinase K (New England Biolabs), ground finely and incubated either at 50 °C for 3 h or 37 °C overnight. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) is added, vortexed and the aqueous phase transferred to a second tube containing 300  $\mu$ L chloroform- isoamyl alcohol (24:1) and the extraction procedure is repeated. The aqueous phase is ethanol precipitated and the resulting DNA pellet is dissolved in 20–50  $\mu$ L sterile water and stored at -20 °C.

[95]**Bertolini *et al.* (2014).** In this method, individual psyllids are immobilized and squashed on nylon or paper membranes with the bottom end of a microcentrifuge tube. Pieces of membrane harbouring the squashed samples are inserted into microtubes containing 100  $\mu$ L distilled water, 0.5% Triton X-100 or

glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA). Samples are then incubated at 100 °C for 10 min as described by Bertolini *et al.* (2014), vortexed and placed on ice until used for real-time PCR.

[96]NAPPO (2012). In this method, one to five adult psyllids are placed into a microfuge tube and homogenized in the tube with a micropestle. DNA extraction is then carried out using the commercial kit Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instructions.

### [97]3.4.3 Conventional PCR

[98]Conventional PCR has proven to be a reliable, specific and sensitive technique for detecting 'Ca. Liberibacter' species in HLB-infected symptomatic trees. Li, Hartung and Levy (2007) determined that there were no significant differences in sensitivity among the conventional PCR methods listed below. All conventional PCR methods can detect 10<sup>-2</sup> dilutions of DNA extracts obtained from 200 mg of midribs from infected plants. Wang *et al.* (2006) quantified the detection sensitivity of conventional PCR as 439 fg/μL DNA extract from infected plants.

#### [99]3.4.3.1 Conventional PCR using the primers of Jagoueix et al. (1996)

[100]Jagoueix *et al.* (1996) used three primers in the same PCR mixture: OA1, OI1 and OI2c (Teixeira *et al.*, 2005a, 2005b). The primer sequences, which are based on the 16S rDNA sequences, are as follows:

[101]OI1 (forward): 5'- GCG CGT ATG CAA TAC GAG CGG CA – 3'

[102]OA1 (forward): 5'- GCG CGT ATT TTA TAC GAG CGG CA-3'

[103]OI2c (reverse): 5'- GCC TCG CGA CTT CGC AAC CCA T-3'

[104]The primer pair OI1/OI2c amplifies 'Ca. L. asiaticus' and 'Ca. L. africanus'; the primer pair OA1/OI2c preferentially amplifies 'Ca. L. africanus'. The sequence of the reverse primer OI2c is the same for all three 'Ca. Liberibacter' species associated with HLB. The sequences of the forward primer OA1 for 'Ca. L. africanus' and OI1 for 'Ca. L. asiaticus' and 'Ca. L. africanus' are identical except that GCA in OI1 is replaced by TTT in OA1.

[105]Although Jagoueix *et al.* (1996) determined that the primer pair OI1/OI2c detects 'Ca. L. asiaticus' and 'Ca. L. africanus', this primer pair does not detect 'Ca. L. americanus' (Li, Hartung and Levy, 2007). No amplification was obtained when this primer pair was tested on *Acinetobacter lwoffii*, *Agrobacterium tumefaciens*, *Citrus tristeza virus*, *Escherichia coli*, 'Candidatus Phytoplasma aurantifolia' (lime witches broom phytoplasma), 'Candidatus Phytoplasma solani' (stolbur phytoplasma), *Spiroplasma citri*, *Xanthomonas campestris*, and *Xylella fastidiosa*. The sensitivity of the method was not quantified, but although amplifications were obtained from 20 mg of infected midribs they were not obtained when lesser amounts of infected midribs were mixed with 1 g of healthy midrib tissue.

[106]The master mix used for this PCR, developed by Jagoueix *et al.* (1996), is described in Table 1.

[107]**Table 1.** Master mix composition, cycling conditions and amplicons for conventional PCR using the primers of Jagoueix *et al.* (1996)

[108]Reagents	[109]Final concentration
[110]PCR-grade water	[111]– <sup>i</sup>
[112]PCR buffer	[113]1×
[114]dNTPs	[115]200 μM
[116]MgCl <sub>2</sub>	[117]2.0 mM
[118]Primer OI1 (forward)	[119]1.0 μM
[120]Primer OA1 (forward)	[121]1.0 μM
[122]Primer OI2c (reverse)	[123]1.0 μM
[124]Taq DNA polymerase	[125]1.5 U
[126]DNA volume	[127]1 μL DNA extract of plant tissue or insect vector
[128]Cycling parameters	[129]
[130]Initial denaturation	[131]94 °C for 2 min
[132]Number of cycles	[133]35
- [134]Denaturation	[135]92 °C for 60 s
- [136]Annealing and elongation	[137]72 °C for 90 s
[138]Final elongation	[139]72 °C for 10 min
[140]Expected amplicons	[141]
[142]Size	[143]1160 bp

[144]<sup>i</sup> For a final reaction volume of 50 μL.

[145]bp, base pairs; PCR, polymerase chain reaction.

#### [146]3.4.3.2 Conventional PCR using the primers of Hocquellet *et al.* (1999)

[147]Hocquellet *et al.* (1999) designed the primers A2 and J5 specifically to detect ‘*Ca. L. asiaticus*’ and ‘*Ca. L. africanus*’. No amplifications were obtained when this method was used on *A. tumefaciens*, *A. lwoffii*, *E. coli*, *Xanthomonas axonopodis* pv. *citri*, *X. fastidiosa* S. *citri*, ‘*Candidatus* Phytoplasma aurantifolia’, and ‘*Candidatus* Phytoplasma solani’ (stolbur phytoplasma). These primers do not detect ‘*Ca. L. americanus*’ (Li, Hartung and Levy, 2007).

[148]The primer sequences, which are based on the DNA sequences of the β- operon, are as follows:

[149]A2 (forward): 5′- TAT AAA GGT TGA CCT TTC GAG TTT- 3′

[150]J5 (reverse): 5′- ACA AAA GCA GAA ATA GCA CGA ACA A-3′

[151]The master mix used for this PCR, developed by Hocquellet *et al.* (1999), is described in Table 2.



[152] **Table 2.** Master mix composition, cycling conditions and amplicons for conventional PCR using the primers of Hocquellet *et al.* (1999)

[153]Reagents	[154]Final concentration
[155]PCR-grade water	[156]– <sup>i</sup>
[157]PCR buffer	[158]1×
[159]dNTPs	[160]200 μM
[161]MgCl <sub>2</sub>	[162]2.0 mM
[163]Primer A2 (forward)	[164]1.0 μM
[165]Primer J5 (forward)	[166]1.0 μM
[167]Taq DNA polymerase	[168]1.0 U
[169]DNA volume	[170]2 μL DNA extract of plant tissue
[171]Cycling parameters	[172]
[173]Initial denaturation	[174]94 °C for 2 min
[175]Number of cycles	[176]35
- [177]Denaturation	[178]92 °C for 20 s
- [179]Annealing	[180]62 °C for 20 s
- [181]Elongation	[182]72 °C for 45 s
[183]Final elongation	[184]72 °C for 10 min
[185]Expected amplicons	[186]
[187]Size	[188]669 bp for 'Ca. L. africanus'
[189]	[190]703 bp for 'Ca. L. asiaticus'

[191]<sup>i</sup> For a final reaction volume of 50 μL.

[192]bp, base pairs; PCR, polymerase chain reaction.

### [193]3.4.3.3 Conventional PCR using the primers of Teixeira *et al.* (2005b)

[194]Teixeira *et al.* (2005a, 2005b) designed the primers GB1 and GB3 specifically for PCR amplification of the 16S rDNA of 'Ca. L. americanus'. The primer sequences are as follows:

[195]GB1 (forward): 5'- AAG TCG AGC GAG TAC GCA AGT ACT-3'

[196]GB3 (reverse): 5'- CCA ACT TAA TGA TGG CAA ATA TAG- 3

[197]The primer pair GB1/GB3 detects only 'Ca. L. americanus' and not 'Ca. L. asiaticus' or 'Ca. L. africanus'. No amplification was obtained when the method was used on *Phytophthora citricola* and *Phytophthora citrophthora*, *X. axonopodis* pv. *citri* strain A, *X. fastidiosa*, (Li, Hartung and Levy, 2007).

[198]The master mix used for this PCR, developed by Teixeira *et al.* (2005b), is described in Table 3.

[199]**Table 3.** Master mix composition, cycling conditions and amplicons for conventional PCR using the primers of Teixeira *et al.* (2005b)

[200]Reagents	[201]Final concentration
[202]PCR-grade water	[203]– <sup>i</sup>
[204]PCR buffer	[205]1×
[206]dNTPs	[207]200 μM
[208]MgCl <sub>2</sub>	[209]2.0 mM
[210]Primer GB1 (forward)	[211]1.0 μM
[212]Primer GB3 (forward)	[213]1.0 μM
[214]Taq DNA polymerase	[215]1.5 U
[216]DNA volume	[217]1 μL DNA extract of plant or insect vector tissue
[218]Cycling parameters	[219]
[220]Initial denaturation	[221]94 °C for 2 min
[222]Number of cycles	[223]35
- [224]Denaturation	[225]94 °C for 45 s
- [226]Annealing	[227]64 °C for 45 s
- [228]Elongation	[229]72 °C for 60 s
[230]Final elongation	[231]72 °C for 10 min
[232]Expected amplicons	[233]
[234]Size	[235]1027 bp

[236]<sup>i</sup> For a final reaction volume of 40 μL.

[237]bp, base pairs; PCR, polymerase chain reaction.

### [238]3.4.4 Real-time PCR

[239]Real-time PCR is an efficient technique for early detection of ‘*Ca. Liberibacter*’ spp. in both symptomatic and asymptomatic trees (Li *et al.*, 2006).

[240]Li, Hartung and Levy (2007) reported that real-time PCR could detect down to 10<sup>-5</sup> dilutions of DNA extracts obtained from 200 mg of midribs from infected plants. Wang *et al.* (2006) quantified the detection sensitivity of real-time PCR as 4.39 fg/μL DNA extract from infected plants. The real-time PCR method of Bertolini *et al.* (2014) showed similar sensitivity for ‘*Ca. Liberibacter*’ spp. detection, as did the method of Li *et al.* (2006).

#### [241]3.4.4.1 Real-time PCR using the primers and probes of Li et al. (2006)

[242]This multiplex, real-time PCR method allows the detection of each of the three ‘*Ca. Liberibacter*’ species in plant tissue and in psyllids. It is based on combinations of three species-specific forward primers, a reverse primer common to all three ‘*Ca. Liberibacter*’ species and a TaqMan probe that anneals to the amplicon of each of the three species associated with HLB. The method can be further multiplexed with internal controls for plant and psyllid tissue. Li *et al.* (2006) observed no substantial differences in Ct values when internal and target primers and probes were multiplexed for the detection of ‘*Ca. Liberibacter*’ spp.

[243]Li *et al.* (2006) determined that the primer–probe set HLBaspr (HLBas/HLBp/HLBr) detects ‘*Ca. L. asiaticus*’ and the primer–probe set HLBafpr (HLBaf/HLBp/HLBr) detects ‘*Ca. L. africanus*’. The primer–probe set HLBaspr can detect ‘*Ca. L. africanus*’ and HLBafpr can detect ‘*Ca. L. asiaticus*’, but with higher Ct values. The primer–probe set HLBampr (HLBam/HLBp/HLBr) detects ‘*Ca. L. americanus*’ but not ‘*Ca. L. africanus*’ or ‘*Ca. L. asiaticus*’. No amplification was obtained when the method was used on *Citrus tristeza virus* and *Curtobacterium flaccumfaciens* strain ER1/6,

*P. citricola* I 22F3, *P. citrophthora* I 1E4, *X. fastidiosa*, *X. axonopodis* pv. *citri* strain A, (Li *et al.*, 2006).

[244] This real-time PCR was further validated in a comparative study of the performance of five real-time PCR methods by Cellier *et al.* (2020) and was shown to be the best method for asiaticus and africanus detection. The sensitivity of the method (the analytical sensitivity) is such that it can detect down to 10<sup>-5</sup> dilutions of DNA extracts obtained from infected *Citrus* plants (Li *et al.*, 2006).

[245] The sequences of the four primers and one TaqMan probe, which are based on the 16S rDNA sequences of the three '*Ca. Liberibacter*' species, are as follows:

[246] Specific to '*Ca. Liberibacter asiaticus*':

[247] HLBas (forward primer): 5' - TCG AGC GCG TAT GCA ATA CG - 3'

[248] Specific to '*Ca. Liberibacter americanus*':

[249] HLBam (forward primer): 5'-GAG CGA GTA CGC AAG TAC TAG - 3

[250] Specific to '*Ca. Liberibacter africanus*':

[251] HLBaf (forward primer): 5'- CGA GCG CGT ATT TTA -3'

[252] Used for all three species:

[253] HLBbr (reverse primer): 5' -GCG TTA TCC CGT AGA AAA AGG TAG-3'

[254] HLBp (hydrolysis probe): 5'-FAM-AGA CGG GTG AGT AAC GCG-BHQ1 -3

[255] The internal control primers and probe to target plant tissue, based on sequences of conserved plant cytochrome oxidase (*COX*) gene from *Citrus* (Li *et al.*, 2006), are as follows:

[256] COXf (forward primer): 5'-GTA TGC CAC GTC GCA TTC CAG A -3',

[257] COXr (reverse primer): 5'- GCC AAA ACT GCT AAG GGC ATT C- 3'

[258] COXp probe: 5'-TET-CAG ATG CTT ACG CTG-BHQ1-3'

[259] The internal control primers and probe to target psyllid tissue, based on sequences of a nuclear gene that codes for a glycoprotein (Manjunath *et al.*, 2008), are as follows:

[260] DCF (forward primer): 5'-TGG TGT AGA TGG TTG TGA TCT GAT GTG -3'

[261] DCR (reverse primer): 5'-ACCGTTCCACGACGGTGA -3'

[262] DCP (hydrolysis probe): 5' -HEX-TGT GGG CGA GGC TAC AGA AC BHQ1-3'

[263] According to Ammar *et al.* (2011), an internal control based on the *D. citri* ribosomal S20 psyllid gene may also be used. The primer and probe sequences are as follows:

[264] Dci-S20-L (forward primer): 5'-GCCCAAGGGCCCCnAATCA-3'

[265] Dci-S20-R (reverse primer): 5'-GGAGTCTTACGGGTGGTTATTCTG-3'

[266] Internal control probe: 5'-FAM -AATGCCCACCAAAGTT- BHQ1-3'

[267] Other real-time PCR master mixes have been shown to work with this method: for example Go Taq Probe qPCR master mix (Promega) (Cellier *et al.*, 2020) and Path-ID qPCR master mix (Ambion) (EPPO, 2014).

[268] The master mix for the primers and probes of Li *et al.* (2006) is described in Table 4.

[269] **Table 4.** Master mix composition and cycling conditions for real-time PCR using the primers and probes of Li *et al.* (2006)

[270]Reagents	[271]Final concentration
[272]PCR-grade water	[273]– <sup>i</sup>
[274]PCR buffer	[275]1×
[276]dNTPs	[277]250 μM
[278]MgCl <sub>2</sub>	[279]6 mM
[280]Primer HLBas (forward)	[281]0.25 μM
[282]Primer HLBam (forward)	[283]0.25 μM
[284]Primer HLBAf (forward)	[285]0.25 μM
[286]Primer HLBr (reverse)	[287]0.25 μM
[288]Probe HLBp	[289]0.15 μM
[290]Internal Primer COXf (forward)	[291]0.30 μM
[292]Internal Primer COXr (reverse)	[293]0.30 μM
[294]Probe COXp	[295]0.15 μM
[296]Taq DNA polymerase	[297]5 U
[298]DNA volume	[299]2 μL DNA extract of plant or insect vector tissue
[300]Cycling parameters	[301]
[302]Initial denaturation	[303]95 °C for 10 min
[304]Number of cycles	[305]40
- [306]Denaturation	[307]95 °C for 20 s
- [308]Annealing and elongation	[309]58 °C for 40 s

[310]<sup>i</sup> For a final reaction volume of 25 μL.

[311]<sup>ii</sup> See page footnote 1.

[312]PCR, polymerase chain reaction.

#### [313]3.4.4.2 *Real-time PCR using the primers and probes of Bertolini et al. (2014)*

[314]This real-time PCR method, which uses universal primers for ‘*Ca. Liberibacter*’ and a TaqMan probe, detects the three ‘*Ca. Liberibacter*’ species associated with HLB and can be performed as a rapid screening test on tissue prints or on leaf or psyllid extracts after a DNA extraction.

[315]The primer sequences, which are based on the 16S rDNA sequences of ‘*Ca. Liberibacter*’ spp, are as follows:

[316]CaLsppF (forward): 5’- GCA GGC CTA ACA CAT GCA AGT -3’

[317]CaLsppR (reverse): 5’- GCA CAC GTT TCC ATG CGT TAT -3’

[318]The sequence for the probe, described by Li *et al.* (2006), is as follows:

[319]HLBp: 5’- FAM-AGA CGG GTG AGT AAC GCG-TAMRA

[320]According to Bertolini *et al.* (2014), the primers and probe used in this method detect all three ‘*Ca. Liberibacter*’ species on *Citrus* spp. The primers CaLsppF and CaL sppR, which are based on the sequence of the most conserved region of the ‘*Ca. Liberibacter*’ spp. genome, were found to detect all the tested ‘*Ca. Liberibacter*’ species associated with HLB from different hosts and origins. No cross-reaction was noticed when the method was tried on other graft-transmitted pathogens of citrus. In further evaluation during a comparative performance study by Cellier *et al.* (2020), false positive amplifications from non-target bacteria were observed. Raising the annealing temperature to 64 °C did reduce some

of this risk. However, because of the residual risk, positive test results using this method can only be considered reliable if they are confirmed by other HLB-specific PCR detection methods.”

[321]The master mix for Bertolini *et al.* (2014) primers and probes is described in Table 5.

[322]**Table 5.** Master mix composition and cycling conditions for real-time PCR using the primers and probes of Bertolini *et al.* (2014)

[323]Reagents	[324]Final concentration
[325]PCR-grade water	[326]– <sup>i</sup>
[327]PCR mix (Path IDqPCR master mix) <sup>ii</sup>	[328]1×
[329]Primer CaLsppF (forward)	[330]0.5 μM
[331]Primer CaLsppR (reverse)	[332]0.5 μM
[333]Probe HLBp	[334]0.1 μM
[335]DNA volume	[336]3 μL DNA extract of plant or insect vector tissue
[337]Cycling parameters	[338]
[339]Initial denaturation	[340]95 °C for 10 min
[341]Number of cycles	[342]40
- [343]Denaturation	[344]95 °C for 15 s
- [345]Annealing and elongation	[346]60 °C for 60 s

[347]<sup>i</sup> For a final reaction volume of 12 μL.

[348]<sup>ii</sup> See page footnote 1.

[349]PCR, polymerase chain reaction.

[350]A diagnostic kit, HLB 100, for use with immobilized plant tissue prints or vector squashes and this PCR method (with lyophilized master mix), is commercially available from Plant Print Diagnostics<sup>1</sup> ([www.plantprint.net](http://www.plantprint.net)). It has been used in surveys in Brazil, Réunion (France) and Spain, among other countries (Bertolini *et al.*, 2014; Siverio *et al.*, 2017).

### [351]3.4.4.3 *Real-time PCR using the primers and probes of Morgan et al. (2012)*

[352]This real-time PCR method was developed for detection of ‘*Ca. Liberibacter asiaticus*’ and uses primers based on the internal 100 bp region of the 132 bp full repeat shared by the high copy *hyvI* and *hyvII* genes.

[353]The primers and probes used are:

[354]LJ900f<sub>f</sub> (forward primer): 5’- GCC GTT TTA ACA CAA AAG ATG AAT ATC -3’

[355]LJ900f<sub>r</sub> (reverse primer): 5’- ATA AAT CAA TTT GTT CTA GTT TAC GAC -3’

[356]Probe LJ900p<sub>p</sub>: FAM-ACA TCT TTC GTT TGA GTA GCT AGA TCA TTG A-Iowa Black FQ

[357]The master mix for the Morgan *et al.* (2012) primers and probes is described in Table 6.



[358]**Table 6.** Master mix composition and cycling conditions for real-time PCR using the primers and probes of Morgan *et al.* (2012)

[359]Reagents	[360]Final concentration
[361]PCR-grade water	[362]– <sup>i</sup>
[363]PCR mix (Fast Universal PCR master mix) <sup>ii</sup>	[364]1×
[365]Primer LJ900f <sub>f</sub> (forward)	[366]0.9 μM
[367]Primer LJ900f <sub>r</sub> (reverse)	[368]0.9 μM
[369]Probe LJ900p <sub>p</sub>	[370]0.5 μM
[371]DNA volume	[372]2 μL DNA extract of plant tissue
[373]Cycling parameters	[374]
[375]Initial denaturation	[376]95 °C for 30 s
[377]Number of cycles	[378]40
- [379]Denaturation	[380]95 °C for 3 s
- [381]Annealing and elongation	[382]62 °C for 30 s

[383]<sup>i</sup> For a final reaction volume of 15 μL.

[384]<sup>ii</sup> See page footnote 1.

[385]PCR, polymerase chain reaction.

#### [386]3.4.4.4 *A combined nested PCR and real-time PCR using the primers and probes of Lin et al. (2010)*

[387]This TaqMan method was developed for detection of ‘*Ca. Liberibacter asiaticus*’. It combines nested PCR and TaqMan PCR in a single tube. The procedure involves two PCR steps using species-specific outer and inner primer pairs with different annealing temperatures, allowing both the first and the second rounds of PCR to be performed sequentially in the same closed tube. The primer–probe sets are as follows:

[388]Inner primers:

[389]Las-I-F (forward): (5'-CGA TTG GTG TTC TTG TAG CG-3')

[390]Las-I-R (reverse): (5'-AAC AATA GA AGG ATCA AGC ATCT-3')

[391]Outer primers:

[392]Las-O-F (forward): (5'-CGG TGA ATG TAT TAAG CTG AGG CGT TCC-3')

[393]Las-O-R (reverse): (5'-TAC CCA CAA CAA AAT GAG ATA CAC CAA CAA CTT C-3')

[394]TaqMan probe:

[395]Las-P: (5' FAM -AATCACCGAAGGAGAAGCCAGCATTACA- MGB 3')

[396]Lin *et al.* (2010) evaluated the specificity (analytical specificity) of the method with over 70 strains of ‘*Ca. L. asiaticus*’ from six different countries and against several non-target pathogens of citrus including ‘*Ca. L. africanus*’, ‘*Ca. L. americanus*’ and ‘*Ca. L. solanacearum*’, *S. citri*, *Xanthomonas citri* subsp. *citri*, *X. fastidiosa*., Only ‘*Ca. L. asiaticus*’ was detected. The sensitivity was estimated as 10<sup>3</sup> copies of target DNA. No other performance data are available.

[397]The master mix for the Lin *et al.* (2010) primers and probes is described in Table 7.

[398]**Table 7.** Master mix composition and cycling conditions for combined nested PCR and real-time PCR.

[399]Reagents	[400]Final concentration
[401]PCR-grade water	[402]– <sup>i</sup>
[403]PCR mix (TaqMan master mix) <sup>ii</sup>	[404]1×
[405]Inner Primer Las-I-F (forward)	[406]2.0 μM
[407]Inner Primer I-R (reverse)	[408]2.0 μM
[409]Outer Primer Las-O-F (forward)	[410]0.05 μM
[411]Outer Primer Las-O-R (reverse)	[412]0.05 μM
[413]Probe Las-P	[414]1.0 μM
[415]DNA volume	[416]2 μL DNA extract of plant tissue
[417]Cycling parameters	[418]
[419]Preincubation	[420]50 °C for 2 min
[421]Initial denaturation	[422]95 °C for 10 min
[423]Number of cycles (1st round)	[424]20
- [425]Denaturation	[426]95 °C for 30 s
- [427]Annealing	[428]67 °C for 45 s
- [429]Elongation	[430]72 °C for 45 s
[431]Number of cycles (2nd round)	[432]35
- [433]Denaturation	[434]95 °C for 30 s
- [435]Annealing	[436]57 °C for 45 s
- [437]Elongation	[438]72 °C for 45 s

[439]<sup>j</sup> For a final reaction volume of 20 μL.

[440]<sup>ii</sup> See page footnote 1.

[441]PCR, polymerase chain reaction.

### [442]3.5 Controls for molecular testing

[443]For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolations and amplifications of the target nucleic acid. For PCR, a positive nucleic acid control (consisting of the target ‘*Ca. Liberibacter*’ species, e.g. ‘*Ca. L. asiaticus*’) and a negative amplification control (no template control) are the minimum controls that should be used. Additional controls may be used for PCR as described below.

[444]**Positive nucleic acid control.** This control is used to monitor the efficiency of PCR amplification. Pre-prepared (stored) nucleic acid, whole genomic DNA or a synthetic control (e.g. cloned PCR product) may be used.

[445]**Internal control.** For conventional and real-time PCR, a plant housekeeping gene such as *COX* (Weller *et al.*, 2000; Li *et al.*, 2006) should be used as an internal control to eliminate the possibility of PCR false negatives resulting either from nucleic acid extraction failure or degradation or from the presence of PCR inhibitors. For an internal control for vectors, a primer–probe set based on the glycoprotein gene in psyllids may be used (Manjunath *et al.*, 2008).

[446]**Negative amplification control (no template control).** This control is necessary for conventional and real-time PCR to rule out false positives resulting from contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture, or sterile phosphate-buffered saline (PBS), is added at the amplification stage.

[447]**Positive extraction control.** This control is used to ensure that the nucleic acid from the target is of sufficient quantity and quality for PCR amplification. Nucleic acid is extracted from known infected host tissue or HLB-positive psyllid DNA.

[448]The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. For PCR, care needs to be taken to avoid cross-contamination resulting from aerosols from the positive control or from positive samples. If required, the positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls may be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

[449]**Negative extraction control.** This control is used to monitor both contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from healthy host plants of the same species but where not available other hosts can be used, such as periwinkle or sweet orange plants grown from seed or healthy psyllids reared on healthy plants.

### [450]3.6 Interpretation of results

#### [451]3.6.1 Conventional PCR

[452]The pathogen-specific PCR will be considered valid only if both the following criteria are met:

- [453]the positive control produces the correct size amplicon;
- [454]no amplicons of the correct size for the bacterium are produced in the negative extraction control or the negative amplification control.

[455]A sample will be considered positive if it produces an amplicon of the correct size. The sample is considered negative when there is no amplification or when a band of the expected size is not produced.

#### [456]3.6.2 Real-time PCR

[457]The real-time PCR will be considered valid only if both the following criteria are met:

- [458]the positive control produces an exponential amplification curve with the pathogen-specific primers and probe
- [459]no amplification curve is seen (i.e. Ct value is 40) either with the negative extraction control or the negative amplification control.

[460]If the *COX* internal control primers are also used, then the negative control (if used), the positive control, and each of the test samples must produce an amplification curve for the test to be considered valid. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction has failed, the DNA has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the nucleic acid has degraded.

[461]A sample will be considered positive if it produces an exponential amplification curve. The cycle cut-off value needs to be verified in each laboratory when implementing the method for the first time. Guidance on how to determine the cycle cut-off value can be found in Chandelier *et al.* (2010).

### [462]4. Identification

[463]The minimal identification requirements are two positive PCR amplifications (from PCR methods described in sections 3.4.3 and 3.4.4) based on sequences of different genes specific for ‘*Ca. Liberibacter*’ spp. or specific for ‘*Ca. L. asiaticus*’, ‘*Ca. L. africanus*’ or ‘*Ca. L. americanus*’.

[464]If the outcome is critical (e.g. post-entry quarantine sample, new record), conventional PCRs that amplify the 16S rDNA gene (section 3.4.3) should be performed and the PCR products sequenced. The primers developed by Jagoueix *et al.* (1996) will amplify a 1160 bp product from ‘*Ca. L. asiaticus*’, or ‘*Ca. L. africanus*’ and primers developed by Teixeira *et al.* (2005b) will amplify a 1027 bp product ‘*Ca. L. americanus*’. Sanger sequencing of these PCR products should be carried out using each primer to generate two independent DNA sequence reads in alternate directions. These sequences should be

aligned to identify conflicting information. Chromatograms should be edited to resolve conflicting signals. If multiple peaks at a nucleotide are observed in the sequences generated using both the forward and reverse primers then the site should be assigned as an ambiguous base (i.e. N = A, C, T or G). The final edited sequence should be at least 900 base pairs (bp) in length for data interpretation. Sequence data can be analysed using the Basic Local Alignment Search Tool (BLASTN), available at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). For species identification the sequence should be at least 99% match to a published authentic sequence.

## [465]5. Records

[466]Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

[467]In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where '*Ca. L. asiaticus*', '*Ca. L. africanus*' or '*Ca. L. americanus*' is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- [468]The original sample should be kept frozen at  $-80^{\circ}\text{C}$ , or freeze-dried, or dried over calcium chloride and kept at  $4^{\circ}\text{C}$ .
- [469]If relevant, DNA extractions should be kept at  $-20^{\circ}\text{C}$  or at  $-80^{\circ}\text{C}$ , and plant extracts spotted on membranes should be kept at room temperature.
- [470]If relevant, PCR amplification products should be kept at  $-20^{\circ}\text{C}$  or at  $-80^{\circ}\text{C}$ .

## [471]6. Contact points for further information

[472]Further information on this protocol can be obtained from:

[473]Laboratory of Plant Pest and Disease, National Agrifood Health and Quality Service (SENASA), Av. Paseo Colón 367, ACD1063, Argentina (Rita Lanfranchi; email: [ritalanfranchi@hotmail.com](mailto:ritalanfranchi@hotmail.com)).

[474]Instituto Valenciano de Investigaciones Agrarias, Carretera de Moncada-Náquera Km 4.5, 46113 Moncada, Valencia, Spain (Ester Marco; email: [emarco@ivia.es](mailto:emarco@ivia.es)).

[475]A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies to the IPPC Secretariat ([ippc@fao.org](mailto:ippc@fao.org)), who will forward it to the Technical Panel on Diagnostic Protocols (TPDP).

## [476]7. Acknowledgements

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[478][Acknowledgements to other contributors to be added later.]

## [479]8. References

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## [534]9. Figures



[535]

[536]**Figure 1.** *Citrus sinensis* (orange) tree on the left not infected with '*Candidatus Liberibacter asiaticus*' compared with tree on the right that is infected with first signs of stunting. Approximately 1 year after planting. Photo courtesy of Greg McCollum, Agricultural Research Service, United States Department of Agriculture, United States.



[537]

[538]**Figure 2.** Four-year-old *Citrus sinensis* (orange) tree declining from Huanglongbing. Small upright leaves near shoot tips (where transmission take place), leaf drop/canopy thinning, and dieback. Photo courtesy of Greg McCollum, Agricultural Research Service, United States Department of Agriculture, United States.



[539]



[540]**Figure 3.** Two adjacent *Citrus sinensis* (orange) trees infected with '*Candidatus Liberibacter asiaticus*' at different stages of Huanglongbing progression. Photo courtesy of Greg McCollum, Agricultural Research Service, United States Department of Agriculture, United States.