Standard Operating Procedure (SOP) for Detecting Citrus Greening Disease using Polymerase Chain Reaction (PCR)

Magh 2080

Government of Nepal
Ministry of Agriculture and Livestock Development

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Office	**National Centre for Fruit Development (NCFD), Kirtipur, Kathmandu**
SOP Number: HLB-1	**Effective Date:** Website publication date of Ministry of Agriculture and Livestock Development (MoALD)
Version Number: 1.0	**Page no:** 25
Review date: -	
Superseded Version Number and Date: -	
Prepared by: NCFD	
Approved by: Ministerial decision (Dated 2080.10.03), Ministry of Agriculture and Livestock Development, Government of Nepal Signature: Name: Dr. Govinda Prasad Sharma Position: Secretary Organization: Ministry of Agriculture and Livestock Development Date: 2080.10.15	

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Contents

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List of Tables

List of Figures

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Abbreviations

Conc.	Concentrate
CTAB	Cetyltrimethyl ammonium bromide
CTV	Citrus tristeza virus
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylene-diamine-tetra acetic acid
EtBr	Ethidium Bromide
e.g.	Example
Fig.	Figure
HCl	Hydrochloric acid
HLB	Huanglongbing
Laf	*Candidatus Liberibacter africanus*
Lam	*Candidatus Liberibacter americanus*
Las	*Candidatus Liberibacter asiaticus*
MoALD	Ministry of Agriculture and Livestock Development
Na_2EDTA	Sodium Ethylene-diamine-tetra acetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCFD	National Centre for Fruit Development
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PPE	Personal Protective Equipment
PVP	Polyvinylpyrrolidone
RNase	Ribonuclease
SOP	Standard Operating Procedure
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
Tris-HCl	Tris hydrochloric acid
UV	Ultraviolet light
vol.	Volume

Measurement

µL	Microliter
asl	Above sea level
g	Gram
g/L	Gram per liter
L	Liter
M	Molar
min	Minutes
mL	Milliliter

MW	Molecular weight
°C	Degree Celsius
rpm	Revolutions per minute
µg/mL	Microgram per milliliter
psi	Pounds per square inch

1. Background

Citrus greening, also known as 'huanglongbing (HLB)' after its Chinese name, "huanglong" means the yellowing of some new shoots in the green canopy, and "bing" means disease. HLB is caused by a phloem limited gram-negative bacteria of α- Proteobacteria class *Candidatus Liberibacter* spp. There are three species of *C. Liberibacter* identified as key pathogens to citrus plants. They are- *Candidatus Liberibacter africanus* (Laf), known as African strain and African citrus psyllid (*Trioza erytreae*) is the vector. This strain is heat sensitive and found at higher elevations (900 m above sea level, asl). *Candidatus Liberibacter asiaticus* (Las), known as Asian strain, is vectored by *Diaphorina citri* (Kuwayama). This strain is more severe, heat tolerant and found at lower elevations (360 m asl), and higher temperatures (30-35°C). *Candidatus Liberibacter americanus* (Lam), known as American strain is also transmitted by *D. citri*. This strain is also heat-tolerant.

Moreover, HLB is a graft transmissible disease. The variability of graft transmission depends upon the plant part used for grafting, the type and amount of tissue used (a single bud or scion stick with multiple buds), age of the tissue, and the season of the year are the major factors that led to success of graft transmission of pathogen (Batool et al. 2007). Greening is also transmitted by dodder (*Cuscuta* spp., family- Cuscutaceae) to non-rutaceous plant such as *Catharanthus roseus* (L.) G. Don (periwinkle- Apocynaceae) and *Nicotiana tabacum* L. cv. 'Xanthii' (tobacco, Solanaceae). Las can multiply and spread within infected *Cuscuta ceanothi* Behr, *Cuscuta campestris* and *C. australis* (Garnier, 2000).

In Nepal, HLB was first reported from Pokhara valley in 1968, then it has been observed in other parts of the country (Ranjit, 2003; Shrestha et al. 2003; Oliya, 2014; Chhetri et al. 2019) leading to a decline and poor-quality citrus fruit in number of orchards throughout the country. So far only the *Candidatus Liberibacter asiaticus* (Las) strain has been reported in Nepal.

1.1 HLB symptoms

Disease symptoms such as yellowing of the apical leaves similar to manganese and iron deficiencies appear on citrus plants in 4 to 5 months after inoculation or blotchy mottle on leaves appears approximately 6 months after grafting or pruning (Razi, et al. 2012). The disease transmission rate varies according to the citrus species and cultivar.

- The appearance of the yellow shoot and die back of the twig/tree (Fig. 1A). Early symptoms of yellowing may appear on a single shoot or branch and takes several years to spread throughout the tree canopy. Mature trees start to decline slowly and eventually become nonproductive after infection.
- Citrus psyllid vector may be seen sucking leaves and stem of the affected tree (Fig. 1B)
- The HLB affected tree shows vein yellowing and random chlorotic blotchy yellow pattern (blotchy mottle) on leaf blade (Fig. 1C, D) whereas, the nutrient-deficient (zinc, iron, and manganese) tree shows the uniform yellow pattern on both sides.
- The blotchy mottle symptom may be confused with other diseases or damage such as severe forms of citrus tristeza virus (CTV), *Phytophthora* root rot, waterlogging, citrus blight, leaf miner tunnels, or citrus stubborn disease. In addition, some leaves may be totally devoid of green or exhibit green island (Dewney et al., 2022).

- Leaves are reduced in shape and size, sometimes vein crocking may be observed, excessive leaf fall and very small and erect types of the new leaf may develop in the affected tree.
- The HLB affected plants bear small fruits, oblong shape, lopsided with curved columella or central core, and the seed if present, is mostly aborted, underdeveloped, and reduced in size (Fig. 1F).
- The fruit exposed to the stem end may show yellow color whereas, the stylar (flower) end shows dull olive (green) color (Fig. 1E) and premature fruit drop occurs. A yellow stain may be present just beneath the peduncle (stem) on a cut fruit.
- Root system is heavily damaged, with 30%–50% root impairment may occur by the time canopy symptoms appear (Atta et al, 2020). In such a tree, root systems are usually poorly developed with relatively few fibrous roots. New root growth is suppressed and the roots often start decaying from the rootlets (Fig. 1G).

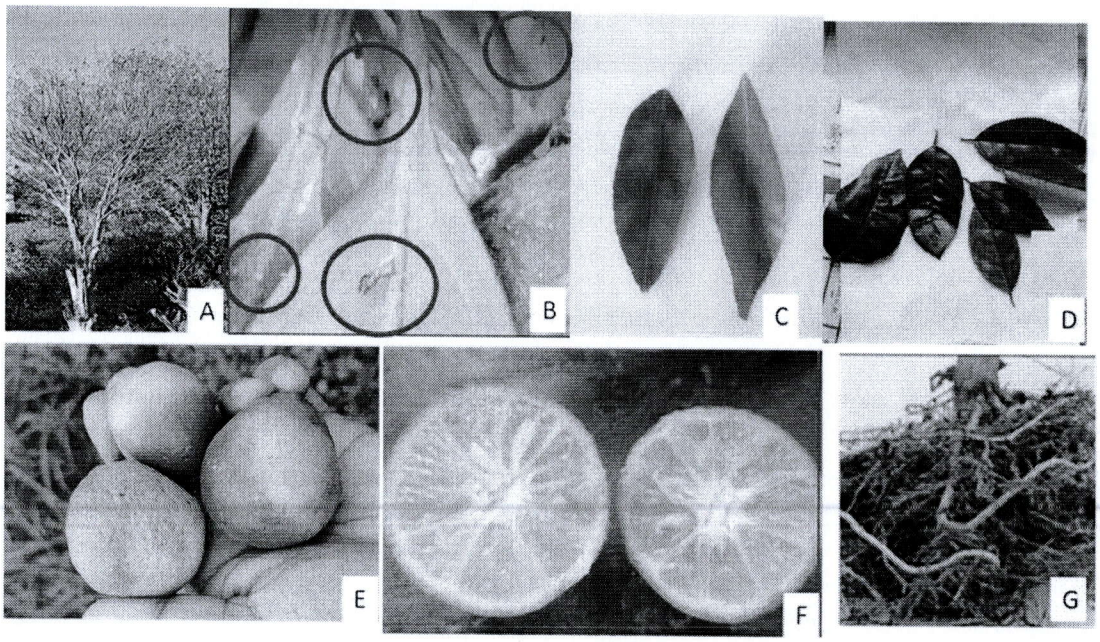

Figure 1. Citrus greening symptoms that can be observed in citrus orchards.
A: Appearance of yellow shoot and die back of tree, B: Asian citrus psyllid (vector) sucking that leaf of the infected tree; C, D: Asymmetrical random chlorotic blotchy yellow pattern (mottle) on leaf blade; E: Presence of yellow and green color on fruit of infected tree; F: lopsided fruit with curved columella and aborted seeds; G: Poorly developed and suppressed type of root system.

1.2 Indexing of HLB

Biological indexing has been a classical method of HLB confirmation using different types of indicator plants. The host range of the *C. Liberibacter* spp. that cause HLB include all citrus species regardless of rootstock. Normally symptoms are severe on sweet orange, mandarins, and their hybrids and moderate on lemon and sour orange. Lime, pomelo, and trifoliate orange are listed as more tolerant, but this does not mean that the bacterium is unable to infect and multiply in these cultivars. In most cases, trees never reach full production, and in the worst case, trees die within 1-2 years of planting. Because HLB also can be transmitted with infected

budwood, the use of certified disease-free planting materials is essential to maximize planting success (Dewdney et al., 2022; Oliya, 2014). The *Psylla* species which transmit HLB, feed on many other rutaceous species. *Diaphorina citri*, the Asian citrus psyllid has a preference for *Murraya* spp. and it has been suggested that *Trioza erytreae's* (the African citrus psyllid) original hosts include *Vepris undulata*, *Clausena anisata* and *Zanthoxylum capense* (Chakrabarty et al.,1976; Moran, 1978).

1.3 Control of HLB

Only way to HLB control is managing the disease using sound integrated pest management. On top of that quarantine is most important for HLB management. Overall, integrated pest management strategies should focus on the use of disease-free planting materials, an optimal nutritional regime, reduction of the inoculum by frequent disease surveys, mass screening by scratch test (starch-iodine reaction), polymerase chain reaction (PCR) diagnosis of the suspected trees/samples, removal of symptomatic trees, and focused management of Las populations (Acharya and Adhikari, 2022; Dewney et al., 2022; Oliya, 2014).

2. Purpose and scope

The main purpose of this SOP is to ensure the proper and accurate diagnosis of citrus greening disease or the HLB and harmonize its diagnosis protocols for related/concerned laboratories for consistency and reliability of diagnosis. The diagnosis method is based on molecular detection method using PCR technology and is applicable to citrus species. The document provides a guidance for the citrus bud wood certification program and describes the steps and requirements for HLB testing facilities involving various steps from sample collection, sample management and storage, laboratory requirement, laboratory test, result reporting, and laboratory safety measures.

3. Sampling and sample management

Sampling is a procedure in which suspected sample is collected outside a laboratory to perform a test. A sample should be representative of the material under test and should be selected based on knowledge of the distribution of the pest to be detected. Sampling usually involves targeting symptomatic plants or plant parts. Appropriate sampling is crucial for HLB detection and identification by chemical, biological, serological, or molecular techniques. Changes to an accepted sampling scheme could impact/affect sound diagnostic protocol generating false positive or false negative results.

3.1 Procedure for sample collection

- Observe the citrus orchard or nursery, and identify the affected tree or saplings (mild, moderate, or severe decline).
- Tag the plant with unique number. Label the sample with the same code assigned to the tree, and collect clean leaves in zip lock bags and label with permanent marker.
- During sample collection, collect symptomatic leaves (blotchy yellow pattern) from all directions of the tree (Fig. 2Λ). Do not collect very young and other disease-affected leaves. If the leaf is dirty and wet, clean and dry it using tissue paper and keep them (almost 12 leaves) in zip lock poly bag and lock the poly bag properly (Fig. 2 B). Leaf having blotchy

mottle pattern is the better choice, such symptoms are the easiest to find from October to March, although they may be present at other times of the year too (Dweney, 2022).

- Make a bundle of twenty such samples (e.g. keep 20 zip lock sample pouch in one packet and tag 1-20 or in sequential order on the outer surface of the bundle, and do the same for the remaining samples).
- Keep the collected sample in an ice box during sample collection and while transporting to the laboratory. Otherwise, collect samples during early morning or evening time, and transport them to the laboratory and kept in refrigerator as soon as possible (within same day). Collected samples should be preserved at normal refrigerator temperature (4°C) for maximum of a day before transporting to the lab.
- Keep record of sample number, collection date, name and address of the orchard owner, citrus species, propagation type of the tree (grafted or seedling), nursery type (screen house or open), age of the tree, any special features of the tree and other detail specified by the laboratory in separate register.
- It is also important to collect vector sample i.e. psylla; to do qPCR for knowing presence or absence of HLB on them.

3.2 Procedure for sample management and storage in the laboratory

- The laboratory should maintain register for different batches of leaf samples used for processing for DNA extraction, PCR amplification and gel electrophoresis. Information related to sample code (original number or code provided by the orchard owner or citrus farmer) and the laboratory code (for the lab record), date of sample collection, the process of sample transport, and the number of samples etc. should be recorded.
- Start work on clean and wide table with adequate space for leaves and other tools. Select symptomatic leaves (depending upon size, 4-5 leaves) from the zip lock bag then, clean the sample properly with cotton dipped in 70-100% ethyl alcohol, separate mid vein from the leaves and cut them into fine pieces using sterilized paper blade or sterile scissors. These fine pieces are packed into labeled aluminum foil. Tag with the same name provided by the service seeker or with lab code, and store at -20°C to -70°C, depending upon the availability of refrigerators in the laboratory (Fig. 2 C, D, E, F, and G).

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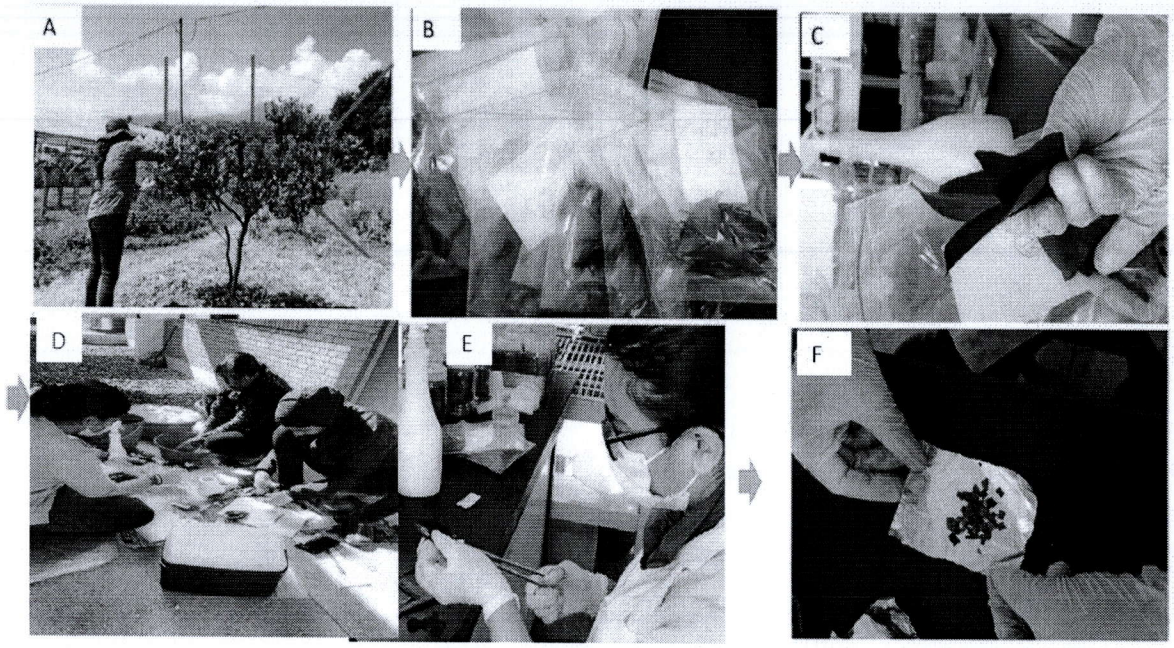

Figure 2. Procedure for sample collection and DNA extraction for detecting Citrus greening disease, A: collection of leaf samples from all directions of the infected/suspected tree, B: sample in zip lock poly bag, C-E cleaning, and management of the collected samples, F: chopping of mid vein of leaf, which is ready for DNA isolation or can be stored for longer time at (-20°C or lower temperature -40°C, -70°C, -86°C).

3.3 Records of sampling

Details of sampling should be recorded and communicated to the appropriate personnel. Records should include the following (Table 1).

Table 1. Detail sampling and information recording

Details of the sample provider	Name: …… Office: ---------- Address: ----------	Designation: …….. Contact No.: …… Other: ----------
Contact detail of the sample collector	Name: ---------- Gender: ---------- Office: ----------	Occupation: ---------- Age: ---------- Contact No.: ---------- Other: ----------
Plant history	Name of species: ----------	Propagation type: ---------- Grafted ( ) Seedling ( )
	Sample from: ---------- screen house ( ) open orchard ( )	Age of the tree: ----------
	Status of the orchard: ----------	Other: ----------
Sampling location	Elevation: ----------	Latitude: ----------
	Longitude: ----------	Municipality: ----------
	Ward no./tole: ----------	District: ----------

	Province: ----------	Other: ----------
Visual symptoms on plants:	Yes ( )	No ( )
If symptomatic plant	Plant canopy: ---------- Yellowing of shoots/twigs yes ( ) No ( )	Die back of twig: yes ( ) No ( )
	Blotchy mottle in the leaf: yes ( ) No ( ) Fruit color: ---------- Off season blooming: yes ( ) No ( ) Premature fruit drop: yes ( ) No ( ) Percentage of fruit drop: ----------	Fruit size: reduced ( ), normal ( ) Lopsided fruit with curved columella (central axis): yes ( ) No ( ) Aborted seed: Yes ( ) No ( )
	Root: (optional):----------	
Sampling procedure:	Sampling date: ----------	Sampling time: ----------
	Tagging of the sample and tree: Yes ( ) No ( )	Orientation of sample leaf: Below canopy ( ) From all direction ( ) Young flush ( )
	Number of leaf in a sample:	Maximum number of sample in a packet: 10 ( ) 20 ( ) other ( )
	Surface sterilization of leaf: ( ) Removing of moisture: ( ) Removing of dust : ( )	Sample transport in ice box: Yes ( ) No ( )
	Means of sample transport:	Date of sample provided to Lab: ----------
Other symptoms on plants:	Nutrient deficiency: Yes ( ) No ( ) Other: ----------	Orchard management: Very good ( ) Good ( ) No ( )
Purpose of sampling	Regular diagnosis ( )	Survey ( )
	Quarantine purpose ( )	Other ( )
At Laboratory	Date of sample (cut pieces of mid rib) storage in -20°C refrigerator or preferably lower temperature (-40° C or -70° C or -86° C) as per the facility available at laboratory	---------- ---------- ---------- ---------- ----
	Date of DNA isolation:	----------
	Sample lay out in record file during agarose gel electrophoresis and PCR reaction preparation	---------- ---------- --

Government of Nepal
Ministry of Agriculture and Livestock Development
Singhadurbar, Kathmandu

Secretary

	Quality test of DNA on (0.8%) agarose gel, using 6x loading dye (2µl) : DNA (4µl)	Yes ( ) No ( )
	Quantity test of DNA	Yes ( ) No ( )
	Date of PCR assay:	----------
	Name of forward primer	----------
	Name of reverse primer	----------
	Remarks on PCR assay result:	Positive ( )
		Negative ( )
	Keep record of DNA image	Folder name:----------
	Keep record of PCR product image	Folder name:----------
	Amplification size of PCR positive sample compared with ladder size	----------
Record the result (both soft/hard copy)		
Do not disclose the result prior to the official decision		
Do not share the data to third party without official permission		

3.4 Laboratory layout, and necessary materials/equipment/chemicals/consumables

The molecular diagnostic laboratory should include the following laboratory set up, instruments and chemicals.

3.4.1 Staff room

The staff room should include the following:

- Sufficient workspace with chairs, tables and computer to maintain records and database of HLB.
- Cloths/Apron hangers,
- Shoe racks,
- Cupboard/locker to store registers and other relevant items

3.4.2 Sample reception and reagent storage room

This room is used for sample reception, sample management and chemical and glassware storage and include the following things and equipment

- Desk for sample reception,
- Reagent storage racks,
- Wide table for sample sorting and preparation
- Normal refrigerator-1 (sample storage until sample management, storage of chemicals)
- Autoclave-1
- Hot air oven-1

3.4.3 Reagent preparation and DNA isolation room

This room is used for chemical preparation and DNA isolation activities and include at least the following equipment/ work environments

- Micro wave oven -1

Government of Nepal Ministry of Agriculture and Livestock Development Singhadurbar, Kathmandu

Secretary

- Hot plate magnetic stirrer with temperature sensor-1
- High precision (four digit) digital weighing balance-1
- pH meter (Desktop- type)-1
- Double distillation system-1 or nuclease free water preparation system
- Single channel micropipette set (0.2-2 µL, 0.5-10 µL, 10-20 µL, 20-200 µL, and 100-1000 µL)- at least one each
- Multi-channel micropipette (0.5-10 µL, 10-100 µL)- at least one each
- Micropipette stands- 2
- Fume hood-1
- Water bath-1
- Mini centrifuge (suitable for Eppendorf tube and PCR strip/tube)-1
- Vortex-1
- Orbital shaker-1
- PCR plate centrifuge (optional)-1
- Liquid nitrogen tank (at least of 35 L, storage type)-1
- Ice buckets with tray-2
- Mortar and pestle (at least 15 sets)
- Water taps and sink-2
- Emergency eye shower-1
- Glassware storage racks
- Working table

3.4.4 Gel electrophoresis room

Gel preparation involves using carcinogenic (e.g. Ethidium bromide) reagents thus all procedures should be conducted in an isolated room.

- Gel electrophoresis set (Power supply and electrophoresis tanks, gel casting trays, combs)
- Gel Documentation System (Gel imager)
- Computer set connected to Gel Documentation system (if necessary, because some gel documentation system has in built touch screen).

3.4.5 PCR room

PCR reagents are prepared in this room thus possible contamination are avoided. This room includes the following equipment/work environment

- Laminar air flow cabinet or Bio Safety Cabinet (Safety level 1)
- PCR Thermal Cycler
- Nano drop Spectrophotometer (to measure DNA quantity/quality)
- Deep Freezer (-20° C, or lower temperature)

3.4.6 Chemical and consumables

3.4.6.1 Chemicals required for DNA isolation

Hexadecyltrimethyl ammonium bromide or Cetyltrimethyl ammonium bromide (CTAB), molecular weight (MW): 336.40 g

- Tris hydrochloric acid (Tris-HCl), MW: 157.60 g
- Sodium chloride (NaCl), MW: 58.44 g
- Ethylenediaminetetra acetic acid (EDTA), MW: 372.24 g
- Sodium acetate trihydrate, MW: 136.08 g
- Polyvinyl pyridine (PVP), MW: 40.0 g
- Tris base, MW: 121.14 g
- Boric acid, MW: 61.83 g
- Hydrochloric acid (HCl),
- Sodium hydroxide (NaOH), MW: 40 g
- DNA isolation kit (optional)
- Agarose (molecular grade)
- Glacial acetic acid

3.4.6.2 Chemicals required for PCR amplification

For PCR based diagnosis of the citrus greening disease, following chemicals are required. The volume/quantity of each chemical depends on the sample target.

- Primers,
- Master mix (including of dNTPS, 10X PCR buffer, Taq polymerase),
- DNA ladder (50 or 100bp)
- Loading dye (6X),
- RNase A,
- Ethidium bromide or safe view- GelRed™

3.4.7 Other consumables and equipment required

HLB testing molecular laboratory requires the following consumables/items the quantity of which depends on the sample testing capacity/target of the laboratory.

- PCR tubes
- PCR plates if samples are more,
- Micropipette tips,
- PCR tube racks,
- DNA storage cryo box,
- ICE maker,
- Cool box to keep primers, Master mix while working,
- Nuclease free water,
- Tissue paper/paper towel,
- Gloves, Masks, etc.

3.5 Procedure for preparation of buffer solutions and chemicals for DNA isolation and visualization

3.5.1 Preparation of 0.5 M EDTA (1000 mL)

Weigh 186.12 g EDTA and add about 700 mL sterile (autoclaved) distilled water. Then adjust the pH 8 by adding NaOH pellets (about 16-18 g is required). EDTA won't dissolve until the

pH is near 8. Bring total volume to 1 L with addition of sterile distilled water. Pour the EDTA solution to reagent storage bottle. And tag the bottle with 0.5M EDTA and date of preparation both in lid and the bottle with permanent marker, then store at room temperature.

3.5.2 Preparation of 3M sodium acetate (100 mL)

Weight 24.61 g of sodium acetate trihydrate and add about 80 mL of sterile distilled water, adjust the pH 5.5 by adding glacial acetic acid. Make the final volume 100 mL by addition of sterile double distill water. Pour the prepared solution in the reagent storage bottle, and tag both in the lid and the bottle as 3M Sodium acetate, pH 5.5. Store in room temperature.

3.5.3 Preparation of 5M NaCl (1000 mL)

Dissolve 292 g of NaCl in 800 mL of sterile double distilled water. Adjust the volume to 1 L with sterile double distilled water. Pour the solution in reagent storage bottle and tag with permanent marker. Store the NaCl solution at room temperature.

3.5.4 Preparation of 1M tris (1000 mL)

Dissolve 121.1g of tris in 700 mL of sterile double distilled water and make the final volume 1000 mL with sterile double distilled water.

3.5.5 Preparation of 1N HCl (1000 mL)

Dissolve 81.8 g of Conc. HCl in 800 mL of autoclaved double distilled water (dd/H_2O) and make the final volume 1000mL.

3.5.6 Preparation of 1N NaOH (1000 mL)

Dissolve 40 g of NaOH in 900mL of sterile distilled water and make final volume 1000 mL with dd/H_2O.

3.5.7 Preparation of 0.5M (pH 8) sodium EDTA (1000 mL)

Add 186.1 g of disodium EDTA.2H_2O to 800 mL of dd/H_2O, stir vigorously on a magnetic stirrer, and adjust the pH to 8 with NaOH (~ 20 g of NaOH). Then store at room temperature.

3.5.8 Preparation of CTAB buffer (1000 mL)

Add 100mL 1M Tris HCl pH 8, 280m 5M NaCl, 40mL of 0.5M EDTA, and 20 g of CTAB. Bring the final volume to 1L with dd/H_2O. Then make the aliquots of 100mL in reagent storage bottle and add 2 g of PVP, stir vigorously on a magnetic stirrer, tag the bottle as CTAB buffer with 2% PVP and store at room temperature.

3.5.9 1X TE buffer (100 mL)

Take 10 mL of 1M Tris HCL (pH 8) and add 2mL 0.5 EDTA and bring total volume to 100 mL with dd/H_2O.

3.5.10 10 X TBE buffer preparation (1000 mL)

Dissolve 108 g of Tris and 55 g Boric acid in 900 mL of dd/H_2O. Add 40 mL 0.5 M Na_2EDTA (pH 8) (alternatively use 9.3 g Na_2EDTA) and adjust the volume to 1L. Store at room temperature.

3.5.11 10 X TAE buffer preparation (1000 mL)

For 1L of 10x solution, add 48.5 g tris in 800 mL of dd/H_2O and after complete dissolve, add 11.4 mL glacial acetic acid and 20 mL 0.5M EDTA (pH 8.0). Then, pour the buffer to reagent storage bottle, close its lid and tag properly (name and preparation date) both on the lid and surface of the bottle.

3.5.12 1X TBE or 1X TAE buffer preparation (1000 mL)

Measure 100 mL of 10X TBE or TAE buffer in measuring cylinder, then add 900 mL of double distilled water, and make the final volume 1000 mL. Then, pour the buffer to reagent storage bottle, close its lid and tag properly (name and preparation date) both on the surface of the bottle.

3.5.13 Preparation of Ethidium Bromide (EtBr) stock solution (10 mL)

Prepare 10 mL of dd/H_2O in a suitable container. Add 10 mg of EtBr to the water. Dissolve the EtBr using a magnetic starrier for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the 10 mg/mL solution to a dark bottle and store at room temperature. *(Note: It is very carcinogenic so use gloves and mask, handle very carefully.)*

3.5.14 Preparation of agarose gel

Weigh out the appropriate mass of agarose and put it into a conical flask. Agarose gels are prepared using a w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The volume of the buffer should not be greater than 1/3 of the capacity of the flask. Add 1X TAE or TBE buffer to the agarose-containing flask, swirl to mix. And then melt the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved. Then let it cool to about 60°C. Add EtBr (0.5-5 µL) from a concentration of 10 mg/mL stock, swirl to mix by avoiding formation of air bubble and pour it to gel casting tray. Allow the agarose to cool on the benchtop. Place the gel tray into the casting apparatus. Pour the molten agarose into the gel mold. And put appropriate comb. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel tank. For example, to prepare 0.8% agarose, weight of agarose: 0.8 g, TAE or TBE required: 100 mL, EtBr: 3-5 µL from 10 mg/mL stock, is required.

3.6 Procedure of DNA isolation

DNA can be isolated using the commercial Plant DNA extraction kit available in the market, in which the protocol of DNA extraction is provided with the kit. Manual method of DNA extraction is using the CTAB method with modification developed by the laboratory. The following procedure of the CTAB method will be followed for DNA isolation (Doyle, 1991) with modification.

- Take approximately 0.2 g of the leaf (mid vein) and grind to fine powder by using mortar and pestle in liquid nitrogen.

- Add 1mL of CTAB buffer (2% CTAB, 0.5M EDTA, 5M NaCl, 1M tris HCL, 0.2% B-mercaptoethanol or 2% PVP) and make a fine paste and transfer to a clean microcentrifuge tube (vol. 1.5 mL).
- Incubate the Eppendorf tubes with the mixture of CTAB buffer and sample at 65° C for 45 min in a water bath and mix the sample gently every 10 min interval.
- Centrifuge at 12,000 rpm for 8 min at room temperature to spin down cell debris.
- Transfer the supernatant (650 µL) to a clean sterilized microcentrifuge tube (vol. 2 mL).
- Add an equal volume of Chloroform: isoamyl alcohol (24:1), mix gently in an orbital shaker for 15 min.
- Again, centrifuge for 5 min at 13,000 rpm and transfer (400 µl) of the upper aqueous phase to a sterile (1.5 µL) micro-centrifuge tube.
- Add ($1/10^{th}$ volume of supernatant) (40 µL) of sodium acetate (3M), followed by the addition of 500 µL of chilled ice-cold absolute ethanol.
- Gently, invert the tubes several times to precipitate the DNA.
- Then, spin the DNA at 13,000 rpm for 2 minutes to form the pellet.
- Discard the supernatant and wash the DNA pellet with ice-cold 70% ethanol (500 µL volume)
- Again spin at 13,000 rpm for 1 minute and discard the ethanol to get rid of salt, this washing step has to be repeated two times to get good quality DNA.
- Then pipette out the ethanol and let the pellet dry for 30 min at 37°C in the incubator or in room temperature.
- Then suspend the dried DNA in 1X TE buffer (150 µL) and store it at 4°C.
- Then check the quality of extracted DNA by gel electrophoresis on agarose gel (0.8%) (As mentioned in 2.4.11) and visualize it using UV light in the Gel documentation system (Fig. 3), and keep the record of DNA in the record file.
- If possible, check the DNA concentration using Nano drop spectrophotometer and equalize the DNA concentration.
- Store the DNA at -20°C for downstream (PCR) assay.

3.6.1 Process of agarose gel electrophoresis

Gel electrophoresis is a technique used to separate DNA fragments according to their size. The detail procedure is given below-

- The well set agarose gel (as described in point 3.5.14) is placed into the electrophoresis tank with sufficient amount of TAE or TBE buffer (use the same buffer that is used in preparation of agarose gel).
- Add loading dye to the DNA samples to be separated (loading dye 2 µL: DNA 4 µL) (this is usually prepared in parafilm). Gel loading dye is typically made at 6X concentration (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel.
- Program the power supply to desired voltage. Attach the lid of the gel box to the power supply.
- Turn on the power supply and verify that both gel box and power supply are working. Remove the lid.

- Slowly and carefully load the DNA sample(s) into the gel (Fig. 3). An appropriate DNA size marker should always be loaded along with experimental samples. Replace the lid to the gel box. The cathode (negative electrode, DNA is loaded) should be closer to the wells than the anode (positive electrode).
- Double check that the electrodes are plugged into the correct slots in the power supply. Turn on the power. Run the gel until the dye has migrated to an appropriate distance (around 45 min).

3.6.2 Observing separated DNA fragments

- When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
- Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
- Remove the gel from the gel tray and expose the gel to UV light. This is done using a gel documentation system. DNA bands should show up as black/white/orange fluorescent bands. Take a picture of the gel (Fig. 3).
- Repeat the DNA isolation of the sample in which the DNA is absent (Fig.3, Lane 5).
- Properly dispose of the gel and running buffer as per the institution's regulations.

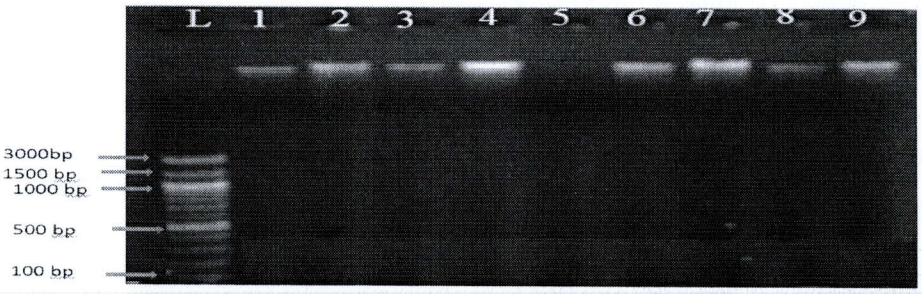

Figure 3. DNA of leaf mid vein visualized on 0.8% Agarose gel. Lane L: 100 bp DNA ladder, Lane 1-9: DNA of different sample. Among nine samples, DNA is absent in Lane 5.

3.7 PCR to detect HLB disease using DNA from leaf mid-vein sample

3.7.1 Primer information

The following primer is used for HLB diagnosis-

Table 2. Name of the Primer, forward and reverse sequence and amplification size of the PCR product.

	Sequence	Remark
Forward Las606_F	5′-GGAGAGGTGAGTGGAATTCCG A-3′	(Fujikawa et al. 2012)
Reverse LSS_R	5′-ACCCAACATCTAGGTAAAAAC C-3′	
Amplification size of HLB positive sample is 500bp (0.5kb)		

3.7.2 PCR reaction preparation

The name of chemicals and quantity required to prepare PCR reaction is given in Table 3.

Table 3. PCR master mixture preparation

S.N.	Chemical	Quantity (for 30.0 µL scale reaction)	Quantity (for 15.0 µL scale reaction)
1.	DNA (template)	2.0 µL	1.0 µL
2.	Forward primer	0.5 µL	0.5 µL
3.	Reverse primer	0.5 µL	0.5 µL
4.	2X PCR master mix	13.0 µL	7.0 µL
5.	Molecular grade water	14.0 µL	6.0 µL
6.	Final volume	30.0 µL	15.0 µL

Note: The quantity of DNA and primer can be adjusting according to the amplification result. If the concentration of DNA is high, you can use 0.5 µL and if the primer dimer is formed you can minimize the primer amount to 0.1 to 0.3 µL.

3.7.3 PCR programming

The basic PCR protocol is as follows-

a. An initial hot start of 95°C for 5 min
b. Denaturation of 94°C for 30-sec
c. Annealing temperature of 55°C for 1 min (35 cycles of step b, c and d)
d. Extension at 72°C for 2 min.
e. A final extension step at 72°C for 10 min.

3.7.4 Visualization of PCR result

PCR result is visualized in 1.5 to 2% agarose gel stained by Ethidium bromide or GelRed™ (safe view). The PCR product is compared with the DNA Ladder marker (Figure 4). The greening positive and negative samples are identified based on the amplification size of the PCR product. The above-mentioned primers will amplify the PCR product of 500 bp and the greening negative sample will not produce a band (Figure 4, lanes 5 and 11). Confusing sample is rechecked and confirmed with the size of positive control and the ladder marker. Then, the result is recorded in the laboratory record file and is provided to the service seeker in the format designed by the laboratory.

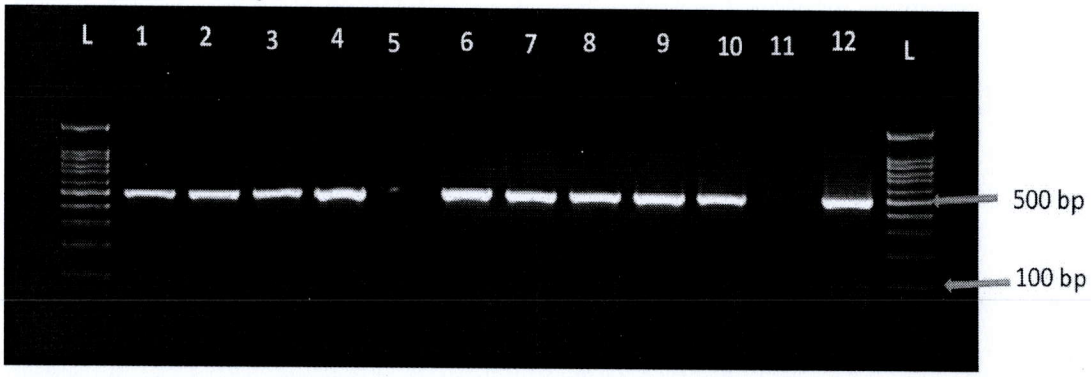

Figure 4. 2% agarose gel image of Asian citrus psyllid detected by greening specific primers forward primer Las606 and the reverse primer LSS. 1-11: suspected samples and 12 is the HLB positive sample. Lanes 1,2,3,4,6,7,8,9,10, showed positive greening result as compared with the Lane 12.

3.7.5 PCR result reporting

The laboratory will provide detailed information about the number of positive (HLB infected) and negative (HLB free) samples in the test report with relevant information of the samples to the concerned person/organization who submitted the sample for HLB test. To receive HLB test report within certain time, the sample provider should contact to the laboratory in advance and get information about the test and acquisition of the subsequent results.

3.7.6 Post HLB test actions

If the laboratory has provided the HLB positive test reports even for a single tree (sample), respective farmer/orchard managers should contact NCFD, Kirtipur or the nearest District Agriculture Office or Horticulture Development Center (Farm Centers or Research Stations) for appropriate HLB management.

4. Fate of the sample

Laboratories must treat a sample once it has been fully analyzed and the final diagnostic report released. Samples may be either disposed of in a manner appropriate to their biosecurity risk or retained for future use.

4.1 Disposal of sample

Before a decision is made to dispose of a sample, the laboratory should decide whether it should be retained as evidence or it should be disposed after autoclaving or by taking appropriate means to minimize the pest risk.

4.2 Sample or specimen retention

A laboratory may choose to retain samples and their related specimens for many reasons. Samples may also be retained in secured manner because of their diagnostic value as reference specimens, or for legal actions. The original sample should be kept at 2 to 8°C for up to 7 days after collection and if a delay in testing or shipping is expected, store specimens at -20°C or lower temperature.

5. Safety and precautions

While working in the molecular diagnostic laboratory, special care should be given as there is always the chances of accidents. Therefore, it is recommended to follow the safe laboratory practices strictly.

- Orientation on laboratory protocols, rules, safety and first aid training must be provided to each staff prior to the start of the work in the laboratory.
- Tagging of the plant, sample, and DNA should be performed very carefully, there should not be any mismatch.
- While performing the wet laboratory work, the chances of mixing the sample are always there, so the laboratory technician/worker/analyzer should be very careful while handling the experiment.
- Laboratory instruments, micropipette tips, and reagents should be clean, and sterile.
- Do not make the laboratory crowded while working, wear lab-coat, mask and gloves.

Government of Nepal
Ministry of Agriculture and Livestock Development
Singhadurbar, Kathmandu

Secretary

- Use fumehood while working with chloroform and isoamyl alcohol as it is harmful to health.
- Do not forget to change the micropipette tips in each sample.
- Proper tagging of the sample in each tube.
- Check the presence of DNA of each sample before PCR assay.
- For PCR reaction preparation, it is suggested to work in Laminar Air Flow cabinet.
- Do not forget to mix the PCR chemicals (master mix, DNA, primer).
- Work quickly, use ice while making PCR reaction.
- The (agarose) gel image of DNA and PCR product should be kept safely with proper (give the lab code) identity in the lab to confirm the result.
- Use positive control/reference, negative control and DNA ladder in each PCR set in each run.
- Do not confuse the result with primer dimer, only the band of target amplicon size is HLB positive.
- Recheck some of the negative samples randomly, sometimes working error (such as missing of addition of primer, or DNA may happen while working).
- Be careful while working with EtBr because, it is highly carcinogenic, toxic and mutagen chemical. It causes eye and skin irritation. Wear gloves at all time, and wash contaminated skin with water. Also dispose the EtBr-stained gel in a separate container, decontaminate it and dispose safely.
- The chloroform mixed samples/tubes should be discarded safely in one container/plastic and should be labelled well.
- All laboratory work must be performed with the proper Personal Protective Equipment (PPE), including goggles or glasses, gloves, lab shoes and lab coat. Additional protection may be necessary, depending on the process.
- All experiments using highly toxic materials must be clearly labeled as such, using a sign or tag, signed and dated.
- Laboratory space should be well ventilated or must have safe exhaust fans.
- Laboratory must have fire extinguisher and first aid kits. Medical emergency number, fire brigade office number shall be clearly displayed.
- Laboratory should have emergency exit.
- Regular calibration of equipment by authentic calibrator is essential.
- If possible, it is better to have molecular grade water purification system in the laboratory.

6. Waste disposal and management

Waste disposal and management is the most important task of each laboratory. The following things should be considered-

- All generators of potentially hazardous wastes must ensure segregation, accurate and complete labeling and safe storage, transport, treatment and disposal of such wastes.
- Wastes should be minimized where possible.
- Waste chemicals and solvents are stored in suitable areas whilst awaiting collection and must not be accumulated.
- Regular disposal from the laboratories must be part of the laboratory program.

- Wastes should be segregated and mixing avoided where possible.
- If you are generating a large amount of one particular type of waste, have a separate residue container for it.
- Ensure the container is not leaking and there is no spillage on the exterior of the container.
- Untrained staff and students are strictly prohibited to handle hazardous wastes and must not be given such responsibility.
- PPE should be a consideration when handling chemical waste. Reference should be made to the Material Safety Data Sheet.
- Broken glassware and solid waste obtained from sample preparation should be segregated and disposed separately.
- Plant material and/or supplies used in the examination and isolation of the suspected sample must be destroyed using a biologically monitored autoclave. The autoclave must be set at a minimum of 15 psi, 121°C for 30 min. All tools and other equipment must be sanitized and/or sterilized before re-use.

7. Distribution and storage of document

The SOP will be distributed as below-

- Ministry of Agriculture and Livestock Development (MoALD), Singhadurbar, Kathmandu
- Department of Agriculture (DoA), MoALD, Hariharbhawan, Lalitpur
- Seed Quality Control Centre (SQCC), MoALD, Hariharbhawan, Lalitpur
- National Center for Fruit Development (NCFD), DoA, MoALD, Kirtipur, Kathmandu
- Central Agricultural Laboratory (CAL), DoA, MoALD, Hariharbhawan, Lalitpur
- Warm Temperate Horticulture Centre, NCFD, DoA, MoALD, Kirtipur, Kathmandu
- Citrus Development Centre, NCFD, DoA, MoALD, Tansen, Palpa
- National Plant Pathology Research Center (NPPRC), Nepal Agricultural Research Council (NARC), Khumaltar, Lalitpur
- Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur
- National Citrus Research Program (NCRP), NARC, Paripatle, Dhankuta
- Provincial Plant Protection Laboratories

The original approved paper SOP will be placed in the approved SOP folder and will be held by Chief, NCFD.

Government of Nepal
Ministry of Agriculture and Livestock Development
Singhadurbar, Kathmandu

Secretary

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Government of Nepal
Ministry of Agriculture and Livestock Development
Singhadurbar, Kathmandu

Secretary