

Citrus greening (*Liberibacter asiaticus*) Detection Kit

Instruction Manual

version 2. 0. 0



NIPPON GENE

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[Read the following instructions before the test]

Thank you very much for purchasing **Citrus greening (*Liberibacter asiaticus*) Detection Kit**. Before using the kit, please confirm the following matters.

Notices for use

1. This kit is designed to detect *Candidatus* *Liberibacter asiaticus*, the causal bacterium-like organism of citrus greening disease (Huanglongbing; HLB), using the Loop-mediated Isothermal Amplification (LAMP) method.
This kit must not be used for clinical diagnosis, therapeutic purposes and the test other than HLB detection.
2. Regarding the storage conditions of the kit, refer to section 2 “Notes” for your reference. The unopened kit is stable for 6 months when stored at -20°C. The kit should be protected from light. Avoid repeated freeze-thaw cycles.
3. Use this kit according to this instruction manual. Nippon Gene Co., Ltd. has no responsibility for any trouble caused by the incorrect use and the different purpose from instructions.
4. Concerning the secondary use of the assay result by the kit, please be notified that the user must be responsible for all the consequential damage from the mishandling or misuse. Nippon Gene Co., Ltd. has no responsibility for any trouble other than that caused by kit defects.
5. Please avoid running electrophoresis, autoclaving of amplified sample after test and positive control to keep the environment free from contaminants.
6. In case of using reagents that are not included in this kit, please follow the notices in the safety instruction of the reagent that you are using. Please do not mix the foreign reagents with the reagents in this kit. Refer to the Safety Data Sheet (SDS) about safe use of this product. If you need the SDS for this product, please contact the contact address on the “Information” section (page 16).
7. This kit is not food. Do not put the reagents into an eye or a mouth. During test, wear a lab coat or gloves and protect the body.
8. LAMP (Loop-mediated Isothermal Amplification) method is developed by Eiken Chemical Co., Ltd.

The LAMP primer set included in this kit, as well as the LAMP-based detection method for citrus greening disease (Huanglongbing; HLB), were developed by the Kyushu Okinawa Agricultural Research Center of the National Agriculture and Food Research Organization (NARO), Japan, under an advanced research program of the Ministry of Agriculture, Forestry and Fisheries (MAFF).

1. About the kit

Product Overview

Citrus greening (*Liberibacter asiaticus*) Detection Kit is designed to detect *Candidatus Liberibacter asiaticus* (*Ca. L. asiaticus*), the causal bacterium-like organism of citrus greening disease (Huanglongbing; HLB), using the Loop-mediated Isothermal Amplification (LAMP) method. The LAMP method is a rapid and easy DNA amplification technique with excellent specificity and sensitivity, and has been applied to the diagnosis of influenza virus infection as well as detection of norovirus, *Legionella* spp., *Salmonella* spp., and enterohemorrhagic *Escherichia coli*. In this kit, a specific region of the *Ca. L. asiaticus* genomic DNA is amplified by LAMP, and the presence of the pathogen is determined based on whether DNA amplification occurs.

The operation required for detection is extremely simple. DNA extracted from test samples using the HLB Extraction Solution provided with the kit is added to the Test Solution (a mixture of HLB Detection Solution, HLB Enzyme Solution, and Fluorescent Detection Reagent), followed by incubation at 65°C for 60 minutes. If *Ca. L. asiaticus* is present in the sample, a characteristic DNA sequence is amplified by the LAMP primer set included in this kit. If the pathogen is absent, no DNA amplification occurs.

For detection of DNA amplification, this kit employs a visual inspection method based on color change of the Fluorescent Detection Reagent. Because DNA amplification and detection are performed in a completely closed single tube, the kit allows safe and rapid detection of *Ca. L. asiaticus* genomic DNA without opening the Reaction Tube after amplification.

Citrus Greening Disease and Its Diagnosis

Citrus greening disease (Huanglongbing; HLB) is a serious citrus disease caused by infection with bacterium-like organisms of the genus *Candidatus Liberibacter*, which localize in the phloem tissue of citrus plants. Infected trees exhibit symptoms such as leaf chlorosis, defoliation, poor fruit coloration, and reduced fruit quality, and eventually decline and die.

HLB is widely distributed in tropical and subtropical regions of Asia, Africa, and the Americas, and is regarded as one of the most destructive diseases affecting citrus production. The disease spreads through grafting and transmission by the Asian citrus psyllid (*Diaphorina citri*) and has become a serious agricultural problem in many countries. In Japan, HLB was first confirmed on Iriomote Island, Okinawa Prefecture, in 1988, and is regulated as an important invasive plant disease under the Plant Protection Act.

Because no resistant cultivars or effective curative treatments have been established, early detection and removal of infected trees are essential to prevent further spread of the disease. Visual diagnosis based on symptoms is difficult, as HLB symptoms closely resemble nutrient deficiency disorders. Moreover, *Ca. L. asiaticus* has not yet been successfully cultured on artificial media, and specific antibodies are not readily available.

Therefore, nucleic acid amplification methods such as PCR and LAMP are widely used for reliable detection.

About LAMP (Loop-mediated Isothermal Amplification) Method

LAMP (Loop-mediated Isothermal Amplification) method allows the whole reaction process, including denaturing, to proceed at a constant temperature in an incubator. Thermal cycling machine is not needed for this kit.

Please refer to the homepage of Eiken Chemical Co., Ltd. about the detailed principle of LAMP method.

2. Reagents provided with the kit

[Kit components (for 48 tests and 192 tests)]

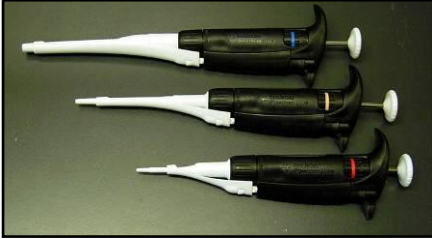
Reagent (tube label)	Form (Top label)	Contents		Storage temperature
		48 tests	192 tests	
Instruction Manual	Booklet	1 booklet	1 booklet	-
Reaction Tube	-	48 tubes	48 tubes x4	Room temperature
HLB Detection Solution	Red	1,050 µl	1,050 µl x 4	-20°C (avoid light)
HLB Enzyme Solution	Yellow	50 µl	50 µl x 4	
Fluorescent Detection Reagent	Purple	50 µl	50 µl x 4	
HLB Positive Control	Gray	50 µl	50 µl x 4	
Mineral Oil	Blue	1,000 µl	1,000 µl x 4	
HLB Extraction Solution	-	6,000 µl x 2	6,000 µl x 8	
HLB Neutralization Solution	Green	1,200 µl x 2	1,200 µl x 8	
Water (Nucleic acid free)	Light blue	1,000 µl	1,000 µl x 4	

Notes

- ◆ This kit can set up test reactions up to 48 samples or 192 samples by preparing a reaction mixture ("Test Solution") for 48 tests or 192 tests in one tube. Please note that the total number of the test that you can do may be less than 48 or 192 if you prepare the test solution over multiple times from one kit. If the test solution is prepared multiple times for fewer tests, reagents may be insufficient.
- ◆ Store reagents other than the Reaction Tube, HLB Extraction Solution, and HLB Neutralization Solution, at -20°C, protected from light, and use within 6 months of delivery.
- ◆ The reagents should be thawed before use, and the remaining reagents should be stored at -20°C again. Repeated freeze-thaw cycle may deteriorate the quality of this kit. Aliquot the contents to several tubes if necessary.
- ◆ HLB Enzyme Solution must be handled with extra care. Do not leave the solution at room temperature or 4°C for a long period of time. Do not freeze it. Under inappropriate temperature conditions, the enzyme may lose its activity.
- ◆ HLB Positive Control is the solution of DNA fragment which contains a DNA sequence specific to *Ca. L. asiaticus* genomic DNA. To avoid cross-contamination, do not spill the solution, and avoid the contact of the used filter tip to the clean equipment and reagents.
- ◆ Consecutive dispensing of the reagent may cause cross-contamination. Use the filter tip as a disposable in every dispensing batch.
- ◆ Handle HLB Extraction Solution (containing sodium hydroxide) and HLB Neutralization Solution (containing acetic acid) with appropriate care. In case of contact with skin or mucous membranes, rinse immediately with plenty of water and take appropriate action according to the SDS.

3. Equipment and reagents not provided with the kit

- Micropipette
(0.5-10 μ l, 10-100 μ l, 200-1,000 μ l)



- Filtered pipette tip (sterilized)



- Microtube for preparation of test solution
(1.5 ml or 2.0 ml)



- Disposable gloves



- Isopropanol

- 80% ethanol

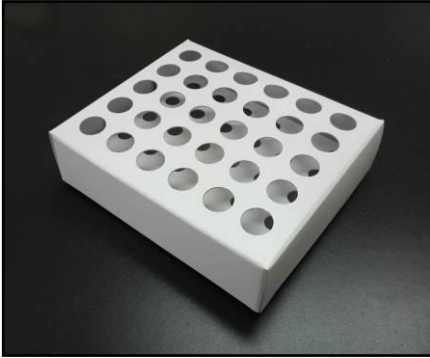
- Incubator
Any equipment that can maintain 95°C, 65°C and 80°C for given time (e.g. water bath, heating block, thermal cycler, air incubator etc.)



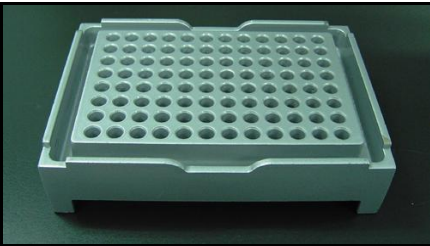
[Equipment which may be useful]

Use these equipments if necessary:

- Microtube rack



- Plate rack



- Vortex mixer



- Centrifuge for microtube



- Centrifuge for test tube



- UV transilluminator

Use when the sample is detected with Fluorescent Detection Reagent.

Transilluminator should output the light with wavelength of 240-260 nm or 350-370 nm.



- UV goggles or face shield

- Float plate

Use when the sample needs to be heated with water bath.

- Tweezers

- Crushed ice

- Vinyl pack for sampling

- 100 mM Tris-HCl (pH 8.0)

- Paper towels

- Wooden toothpicks

- Proteinase K

- High-speed centrifuge

4. Instructions

Simplified Protocol

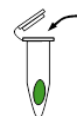
1. Sample preparation

Punch out the midrib portion of a citrus leaf sample using a biopsy punch or similar tool.



2. DNA extraction

Place the leaf disc into a 1.5 ml microtube and add 250 µl of HLB Extraction Solution.



3. Heat treatment

Incubate the tube at 95°C for 5 minutes.

4. Neutralization

Transfer the aqueous phase to a new tube and add 50.0 µl of HLB Neutralization Solution.

5. DNA precipitation

Add 300 µl of isopropanol and mix thoroughly.

6. Centrifugation

Centrifuge at 15,000 rpm for 15 minutes at 4°C.

7. Washing

Wash the pellet with 80% ethanol and air-dry.

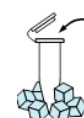
8. DNA dissolution

Dissolve the DNA pellet in 50.0 µl of Water (Nucleic acid free).

9. Preparation of Test Solution

Prepare the Test Solution in bulk for the required number of tests.

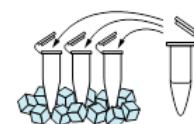
Reagent	1 test	8+1 tests*	24+1 tests*
HLB Detection Solution	21.0 µl	189.0 µl	525.0 µl
Fluorescent Detection Reagent	1.0 µl	9.0 µl	25.0 µl
HLB Enzyme Solution	1.0 µl	9.0 µl	25.0 µl
Total	23.0 µl	207.0 µl	575.0 µl



* Prepare the test solution for the number of the samples that you test + 1 test, so that you have enough volume for the test solution of all samples.

10. Dispense Test Solution

Transfer 23.0 µl aliquot of the test solution into the Reaction Tube.



11. Sample addition

Add 2.0 µl of DNA sample to each Reaction Tube.

* Always add the HLB Positive Control last.

12. Mineral Oil overlay (if required)

Overlay 20.0 µl of Mineral Oil on each reaction.

* If evaporation of the test solution is expected, addition of Mineral Oil is required.

* When using a thermal cycler equipped with a heated lid, Mineral Oil is not necessary.

13. Test reaction (DNA amplification)

Incubate at 65°C for 60 minutes.

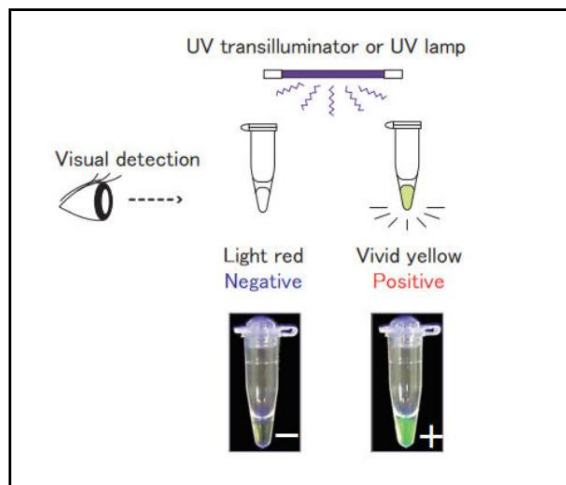
14. Reaction termination (Enzyme inactivation)

Incubate at 80°C for 2 minutes to terminate the reaction.

15. Judgement

Judge the result by visual inspection or under UV illumination.

- Negative: light red color
- Positive: vivid yellow-green color



Preparation and Precautions Before Detection

Sample preparation

■ Control

This kit includes an HLB Positive Control to verify the performance of the detection assay. To ensure the validity of the test results, prepare a Positive Control reaction containing the HLB Positive Control and a Negative Control reaction containing nuclease-free water.

■ Preparation of DNA samples

DNA extracted from citrus plants or Asian citrus psyllids can be used as a sample. This kit provides reagents (HLB Extraction Solution and HLB Neutralization Solution) and a simple protocol for DNA extraction from citrus leaf samples. Please prepare DNA samples prior to starting the test.

Important

When DNA is extracted from frozen citrus leaf samples, polysaccharide contamination may increase and inhibit subsequent DNA amplification. This may result in false-negative results. When DNA is extracted using commercial plant DNA extraction kits or the CTAB method, prepare leaf samples containing the midrib portion. Commercial kits such as *ISOSPIN Plant DNA (Nippon Gene)*, *Nucleon PhytoPure (Cytiva)*, *DNeasy Plant Mini Kit (Qiagen)*, or equivalent products may be used.

Ensure that the DNA concentration is not lower than 5 ng/μl.

Avoid using samples containing chelating agents such as EDTA at concentrations exceeding 1 mM, as this may interfere with the reaction.

For dilution or dissolution, Water (Nucleic acid free) or 10 mM Tris-HCl (pH 8.0) is recommended.

Equipment preparation

■ Incubator

Turn on the power of an incubator and set the required temperature. In case of using a water bath and a heat block, it might take time to reach the target temperature, so pre-heat the device and confirm the temperature with a thermometer. In case of using an air incubator, the temperature alters largely when the cabinet door is open. The door opening operation should be quickly done when the sample is set in an air incubator.

■ Other equipment

Equipment	Instruction
Micropipette	Use micropipettes exclusively in designated LAMP working area. If micropipettes are used in other areas, ensure that DNA decontamination is performed before returning them to their original location.
Microtube rack	Use microtube rack exclusively in designated LAMP working area. If racks are used in other areas, ensure that DNA decontamination is performed before returning them to their original location.
Microtube	Select gamma-ray sterile, nucleic acid-free, and nuclease-free grade microtube.
Filtered pipette tip (sterilized)	Select gamma-ray sterile, nucleic acid-free, and nuclease-free grade pipette tip with hydrophobic filter and unpack at working area. Consecutive dispensing of the reagent with one tip may cause cross-contamination, use the filter tip as expendables in every dispensing batch.
Writing materials	Use solely in each working area and ensure a dedicated space for the documents brought in the area.
Disposable gloves	Use gloves as expendables, change them when the contamination is suspected.
Laboratory coat	Use solely in each working area, be aware of potential contamination from sleeves or cuffs.

Testing Environment

Because LAMP method is the DNA amplification technology with excellent sensitivities, accurate test results may be difficult to obtain if the testing environment is contaminated with HLB positive control or amplified samples after test. For the handling of the samples, take extra care to avoid contact of the positive control and samples with the laboratory coats and equipments. Exchange of clothes is also strongly recommended. To prevent the false results after the test, the used tips, microtubes, and amplified sample after test should be packed together in doubled plastic bags. Please avoid running electrophoresis, autoclaving of amplified sample after test and positive control.

■ Working area

Assign a clean booth or working bench which has not been used for nucleic acid extraction and amplification (which has not been contaminated with nucleic acid) as dedicated reagent preparation area. Prepare the test solution only at the reagent preparation area. Do not use HLB positive control, any solution or reagents that may become a template for LAMP method. Separate the dedicated nucleic acid handling area from reagent preparation area. Addition of sample and HLB positive control must be done only at nucleic acid handling area as the dedicated working area.

■ Nucleic acid decontamination operation

Keep the equipment always clean. Wash the equipment with large amounts of tap water to dilute and wash off the nucleic acids on the surface if possible.

If it is suspected to have nucleic acids contamination on the surface of goods, especially after handling highly concentrated nucleic acids, it is recommended to decontaminate the testing environment from nucleic acids with 1% sodium hypochlorite aqueous solution. Please ensure adequate ventilation during use because sodium hypochlorite generates chlorine gas. Since aqueous sodium hypochlorite solution is corrosive on metals, it is necessary to wipe immediately the chlorine content from surface when used on metals. Sodium hypochlorite aqueous solution can easily deteriorate under high temperature environment, so pay attention to the days elapsed and storage temperature since 1% solution has been prepared.

<Protocol for nucleic acid decontamination using 1% sodium hypochlorite aqueous solution>

- i) Wear disposable gloves on hands.
- ii) Prepare 10,000 ppm (1%) sodium hypochlorite aqueous solution.
- iii) Gently wipe the working bench and equipment with a paper towel moistened with sodium hypochlorite aqueous solution followed by wiping with a paper towel moistened with 70% ethanol.
- iv) For non-metal equipment, soak the equipment in sodium hypochlorite aqueous solution for more than one hour followed by rigorous rinsing with water and drying.
- v) Keep working bench and equipment always clean and perform wiping by sodium hypochlorite aqueous solution regularly.

Detailed Protocol

DNA Extraction

This kit provides reagents (HLB Extraction Solution and HLB Neutralization Solution) and a simplified extraction protocol for the rapid extraction of DNA from citrus leaf samples (midrib region).

If DNA samples have already been extracted and purified using a commercially available plant DNA extraction kit or the CTAB method, please proceed directly to the **Test reaction** section.

< I. DNA extraction from citrus leaf samples >

I-a. DNA Extraction

Place citrus leaf discs (midrib portion, approximately 6 mm) into a 1.5 ml tube.

Up to five leaf discs collected from the same or different leaves can be processed together in a single tube.

Add 250 µl of HLB Extraction Solution, mix gently, and incubate at 95°C for 5 minutes (10 minutes if using an air incubator).

Spin down and transfer the aqueous phase to a new tube.

Important

Do not grind the sample.

Note that the HLB Extraction Solution contains sodium hydroxide; handle with extreme care.

If it comes into contact with skin or mucous membranes, rinse immediately with a large amount of tap water and take appropriate action.

I-b. Neutralization

Add 50.0 µl of HLB Neutralization Solution.

I-c. Purification

Add 300 µl of isopropanol, mix, and centrifuge at 15,000 rpm, 4 °C for 15 minutes.

Remove supernatant, wash pellet with 80% ethanol, air-dry.

Dissolve DNA pellet in 50.0 µl of Water (Nucleic acid free) or 10 mM Tris-HCl (pH 8.0).

< II. DNA Extraction from Asian Citrus Psyllids >

II-a. DNA Extraction

Add 100 µL of buffer solution containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 25 mM NaCl, and 5% Proteinase K to a 1.5 mL tube containing Asian citrus psyllids and homogenize using a sterile toothpick or a similar sterile implement.

Important

Do not grind the sample.

Note that the HLB Extraction Solution contains sodium hydroxide; handle with extreme care.

If it comes into contact with skin or mucous membranes, rinse immediately with a large amount of tap water and take appropriate action.

II-b. Heat Treatment

After incubation at 55 °C for 60 minutes, perform heat treatment at 94 °C for 15 minutes.

II-c. Purification

Transfer the supernatant to a new 1.5 mL tube and use it as the DNA sample.

Test reaction

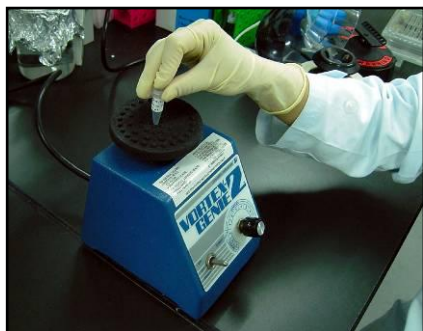
Thawing of reagents

Thaw the following reagents at room temperature:

- HLB Detection Solution
- Water (Nucleic acid free)
- HLB Positive Control
- Mineral Oil

Take out HLB Enzyme Solution and Fluorescent Detection Reagent from the freezer just before use because they do not freeze at -20 °C.

Mix and spin down



Gently tap the tubes several times (hereinafter referred to as “tapping”) or vibrate the tubes 3 times for approximately 1 second on a vortex mixer. After the mixture becomes homogenous, centrifuge the tubes to collect the solution in the bottom of the tubes (hereinafter referred to as “spin down”), and then put the reagents on ice.

Preparation of the Test Solution

Mix HLB Detection Solution, Fluorescent Detection Reagent, and HLB Enzyme Solution in a sterile microtube and spin down. Keep on ice.

Reagent	1 test	8+1 tests *
HLB Detection Solution	21.0 µl	189.0 µl
Fluorescent Detection Reagent	1.0 µl	9.0 µl
HLB Enzyme Solution	1.0 µl	9.0 µl
Total	23.0 µl	207.0 µl



* Prepare the test solution for the number of samples that you test + 1 test, so that you have enough volume for the test solution of all samples.

Important

Consecutive dispensing of the reagent with one tip may cause cross-contamination, use the filter tip as expendables in every dispensing batch.

Because the HLB Enzyme Solution is highly viscous, please take care during dispensing to prevent excessive adhesion around the filter-equipped tips.

Perform a spin down before use.

Consecutive dispensing of the reagent with one tip may cause cross-contamination, use the filter tip as expendables in every dispensing batch.

Distribution and sample addition



Pick a Reaction Tube out from the bag with a clean tweezer. Dispense 23.0 µl of Test Solution into each Reaction Tube.

Important

Only use the specified Reaction Tube which is attached to this kit. Use of different volumes, shapes, and materials may cause misjudgment.

Sample addition

After adding 2.0 µl of the DNA sample to the Test Solution, overlay the solution with approximately 20.0 µl of the Mineral Oil supplied with this kit to prevent concentration of the Test Solution due to evaporation, and then close the cap. *Evaporation-induced concentration of the Test Solution will markedly reduce the efficiency of the assay; therefore, please take care to prevent evaporation.* When using a thermal cycler equipped with a heated bonnet (heated lid) as the incubator, the addition of Mineral Oil is not required.

Important

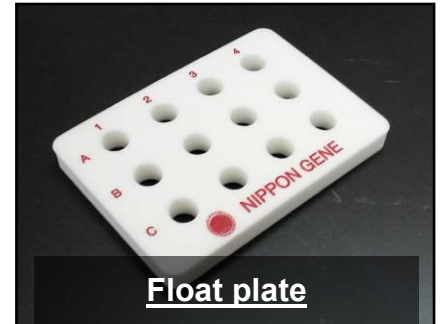
When using DNA samples extracted and purified with commercial plant DNA extraction kits or by the CTAB method, ensure that the DNA sample concentration is not less than 5 ng/µl.

Incubation

Close Reaction Tube and mix. The reagents by tapping or vibrating on a vortex mixer for about 1 second x 3 times, and spin down the tube. Incubate Reaction Tube at 65°C for 60 minutes in the incubator (Air incubator, heating block, or water bath).

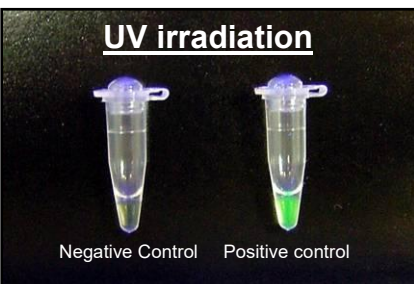
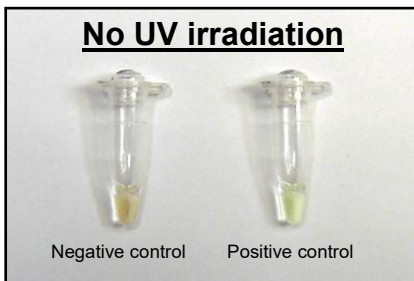
In case of using water bath, use a float plate to prevent the Reaction Tube tilted on the water surface.

In case of using water bath, use a float plate to prevent the Reaction Tube tilted on the water surface.



Judgement

Judgement of success or failure of the test



After 60 minutes incubation, terminate the reaction by heating for 2 minutes at 80°C. Fluorescence Detection Solution appears light red before the reaction. If *Ca. L. asiaticus* exists, it turns vivid green under UV irradiation.

In this procedure, UV transilluminator (wavelength: 240 - 260 nm or 350 - 370 nm), UV goggles, or a face shield is needed.

First, confirm that the Positive Control Solution emits fluorescence and the Negative Control Solution does not emit fluorescence. In case if this condition is not met, the result is invalid and the cause should be investigated.

Important

To avoid false results, judge the result immediately after the reaction is completed.

Judgement of the sample

After the control solutions have been evaluated and the test is confirmed as valid, evaluate the results of sample. If the Test Solution emits the fluorescence at the same level as the Positive Control Solution under UV irradiation, the sample may contain *Ca. L. asiaticus*. If **no** fluorescence is detected under UV irradiation, the sample does not contain *Ca. L. asiaticus*.

[Tips of judgement]

Test Solution emits fluorescence clearly

Samples that exhibit distinct and clear fluorescence are judged as:

“*Ca. L. asiaticus* Positive = Citrus Greening Disease Positive.”

Even if the fluorescence intensity is weak, samples that show a detectable difference when compared with the negative control solution must be retested to confirm the result.

Test Solution does not have any difference in fluorescence compared with Negative Control Solution

Samples that show no significant difference in fluorescence intensity when compared with the negative control solution are judged as:

“*Ca. L. asiaticus* Negative = Citrus Greening Disease Negative.”

Depending on the infected part of the citrus tree or seedling and the infection level, the pathogen may not be detected even when using this kit.

Therefore, carefully observe disease symptoms, and if infection is suspected, collect leaves from multiple locations of the same plant and perform periodic testing.

5. Troubleshooting

If you experience trouble with this kit, check the items below and try the solutions.
Consult Nippon Gene Co., Ltd. for further questions.

Problem	Possible cause and solution
Control test solution does not give the right coloring.	A. Too much HLB Positive Control added to the test solution There are some cases that efficiency of test reaction decreases when too much HLB Positive Control is added to the reaction. Please follow the instructions for the correct amount of addition. B. Reagents or testing environment are contaminated with nucleic acid If the negative control testing solution gives color change, template DNA contamination is suspected. Check the reagents and testing environment for contamination, and perform cleaning procedures using a 1% sodium hypochlorite aqueous solution to completely remove the contaminants. After decontamination, repeat the test. C. Chelate compounds or metal ion in the sample The Fluorescent Detection Reagent emits fluorescence when chelate compounds such as EDTA exist in the reaction. On the other hand, if a lot of metal ion is present in reaction, the fluorescence is inhibited thus it would be difficult to judge the result. D. Reaction temperature and operating procedure do not correct Confirm that there is no problem with the testing process.
Irregular coloring of fluorescent detection solution	A. Judgement not immediately after test reaction has ended Fluorescent Detection Reagent irregularly gains or loses its coloring when left in room temperature for long time. Follow the instructions for storage and handling of the solution.
Test solution has evaporated.	A. The Reaction Tube not heated homogeneously Water bath and heat block may not have heated the Reaction Tube homogeneously so that the Test Solution would be concentrated because of evaporation. In such cases the reactivity efficiency goes down. Make sure that Mineral Oil to be added to the Test Solution.

Problem	Possible cause and solution
Judging fluorescence is difficult.	A. UV lamp wavelength is not optimal. UV lamp emitting light wavelength of 240-260 nm or 350-370 nm is necessary for the detection. In case of the wavelength of the light is 320 nm, be notified that negative sample could emit fluorescence (false-negative).
Insufficient Reagent Volume	A. Reagent has splashed or adhered to the inner wall of the tube Before use, perform a spin down to collect the reagent at the bottom of the tube. B. Reagent has evaporated during storage After use, ensure that the cap is tightly closed to prevent evaporation.

6. Supplementary data

[Quality control]

Quality control testing is performed using this kit as follows:

- DNA amplification is carried out using 2.0 µl of the HLB Positive Control as template DNA in a 25.0 µl reaction volume.
Fluorescence is confirmed after incubation at 65°C for 60 minutes.
- DNA amplification is also performed using 2.0 µl of Water (Nucleic acid free) as template DNA in a 25.0 µl reaction volume.
No fluorescence is observed under the same reaction conditions.

[Copy number of HLB Positive Control]

The HLB Positive Control contains approximately 1×10^5 copies of the target DNA sequence per 2.0 µl.

7. References

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