Phytoplasma Universal Detection Kit

Instruction Manual

version 2.0.0

NIPPON GENE

Phytoplasma Universal Detection Kit
Instruction Manual Version 2.0.0

[Read the following instructions before the test]

Before using the kit, please confirm the following notices.

Notices for use

- This kit offers detection of all Phytoplasma in the class *Mollicutes* from plant samples using LAMP, means Loop-mediated Isothermal Amplification. This kit must not be used for clinical diagnosis, therapeutic purpose and the test other than Phytoplasma detection.

- Concerning the storage procedure of the kit, please read section 2 “Notes” for your reference. The unopened kit is stable at -20˚C for 6 months. It should be kept in dark. Avoid repeated freezing and thawing.

- Use this kit according to this instruction manual. Nippon Gene Co., Ltd. has no responsibility for any trouble caused by the different use and the different purpose from instructions.

- 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.

- Please avoid running electrophoresis, autoclaving of amplified sample after test and positive control in order to keep the environment free from contaminants.

- 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. MSDS is exhibited in the homepage of Nippon Gene Co., Ltd.
  URL; **http://nippongene-analysis.com/**

- 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.

- Eiken Chemical Co., Ltd. owns the patent right for execution of Loop-mediated isothermal Amplification. Nippon Gene Co., Ltd. has been granted the license to develop, manufacture and sell the kit for Phytoplasma identification.

Table of contents

The primer set of this kit and the Phytoplasma detection technology using the primer set and LAMP method are developed by The University of Tokyo, Plant Clinic.

1. About the kit

Product Overview

Phytoplasma Universal Detection Kit offers detection of all Phytoplasma in the class *Mollicutes* inclusively from plant samples using Loop-mediated Isothermal Amplification (LAMP) method. LAMP method is fast and easy DNA amplification method which is also used for the diagnosis of influenza virus and detection of norovirus, *Legionella* sp., *Salmonella* sp., and Verotoxin-producing *E. coli*, exhibiting excellent specificity and sensitivity. In this kit, a part of Phytoplasma DNA amplifies using LAMP method, and infection of Phytoplasma can be judged if the amplification occurs or not.

The operation needed for the detection is extremely easy, simply to mix the DNA extracted from plant sample to the test solution (mixture of Phytoplasma Detection Solution, Phytoplasma Enzyme Solution, and Fluorescent Detection Solution) in a sample tube, and keep the tube at 64 degree Celsius for 60 minutes. The existence of Phytoplasma in the plant can be determined from whether the specific sequence amplifies with LAMP primer set or not.

For detection of DNA amplification, this kit utilizes visual inspection of fluorescence emitted from the solution after the whole reaction, which means that the DNA amplification and detection can be done in one closed tube. Therefore, the amplification of Phytoplasma DNA can be detected safely in a short period of time.

The “List of Phytoplasma diseases in Japan” is available at our homepage for your information.

The kit may not detect the complete absence of Phytoplasma depending on the plant species and Phytoplasma species. If Phytoplasma infection is still suspected when not detected, please consult with The University of Tokyo, Plant clinic.

Diagnosis of Phytoplasma Diseases

Phytoplasmas are plant pathogenic bacteria hosting on the phloem of plants, which were first discovered in Japan, 1967. They infect over 700 plant species worldwide, cause numerous symptoms as yellows, stunt, dwarf, proliferation, phyllody, witches’ broom, and are transmitted by insect vectors to many economically important crops, fruit trees and ornamental plants.

In order to prevent the devastating damage to the plant, it is necessary to provide effective means for controlling Phytoplasma such as removal of infected plant. However, since Phytoplasma is difficult to cultivate, it has not been provided the convenient way to diagnose the Phytoplasma infection. This kit can detect Phytoplasma inclusively, with no means of difficult operation such as conventional culture methods.

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

Eiken GENOME SITE; http://loopamp.eiken.co.jp/e/

About The Synthetic Oligonucleotide Included in This Kit

The primers included in this kit are all “Reliable & Traceable oligo”. “Reliable & Traceable oligo” is one of the highly reliable oligonucleotide series manufactured under control of ISO 13485:2003 certification by Nippon Gene Material Co., Ltd. The oligonucleotides are all produced under dedicated positive pressure environment, with checklists to control process and ensure the full traceability of the production.

Please refer the homepage of Nippon Gene Material Co., Ltd. about the details of “Reliable & Traceable oligo”.

Nippon Gene Material Co., Ltd.; http://www.nippongenematerial.com/

Refer

2. Reagents provided with the kit

[Kit components (for 48 tests)]

Reagent (tube label)	Form (top label)	Contents 48 tests	Storage temperature
Instruction Manual	Booklet	1 booklet	Room temperature
Test tube	Bag	50 tubes	Room temperature
Phytoplasma Detection Solution	Red label	1,050 µl	-20˚C (avoid light)
Phytoplasma Enzyme Solution	Yellow label	50 µl	-20˚C (avoid light)
Fluorescent Detection Solution	Purple label	50 µl	-20˚C (avoid light)
Phytoplasma Positive Control	Gray label	50 µl	-20˚C (avoid light)
Mineral Oil	Blue label	1,000 µl	-20˚C (avoid light)
Phytoplasma Extraction Solution	Bottle	5,000 µl	-20˚C (avoid light)

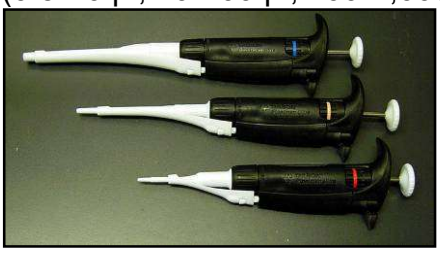
Notes

- This kit can set up the test reactions up to 48 samples by preparing test solution for 49 tests in one tube. Prepare the test solution for the number of the samples that you test + 1 test, so that you have just enough volume for the test solution of all samples. Please be noticed that the number of the test that you can do will be less than 48 if you prepare the test solution over several times from one kit.
- If the water drop is attached to the test tube or bag, completely dry it before you open and use.
- Store all reagents other than Instruction Manual and Test tube at -20˚C. Protect them from light. Use until 6 months upon arrival date.
- The reagents should be thawed each time when used, and the remaining should be stored at -20˚C again. Repeating freeze and thaw may deteriorate the quality of this kit. Aliquot the contents to several tubes if necessary.
- Phytoplasma Enzyme Solution must be handled with extra care. Do not leave it at room temperatures or 4˚C for a long period of time. Do not freeze it. Under inappropriate temperature condition, the enzyme may lose activity.
- Phytoplasma Positive Control is the solution of DNA fragment which contains a DNA sequence specific to Phytoplasma genomic DNA. To avoid cross-contamination, do not spill the solution, and avoid the contact of 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.
- Mineral Oil might belong to the hazardous substance depending on the local administration. Please refer the local regulation concerning hazardous substances.

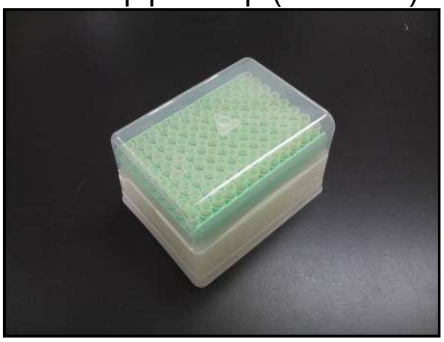
3. Equipment and reagents not provided with the kit

[Required equipment and reagent]

- Micropipette
  (0.5-10 µl, 10-100 µl, 200-1,000 µl)



- Filtered pipette tip (sterilized)



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

- Disposable gloves

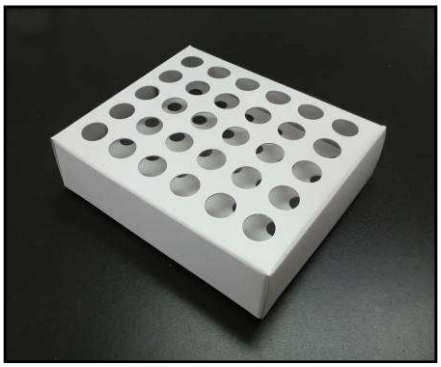
- Incubator
  Any equipment that can maintain 64˚C for given time (e.g. water bath, heating block, thermal cycler, air incubator etc.)

- Razor
- Paper towel
- Tweezers
- Crushed ice

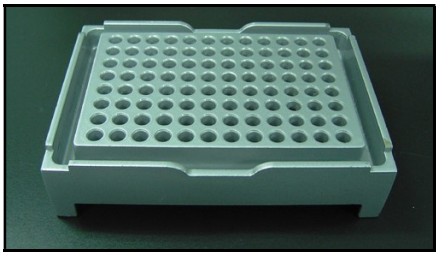
[Equipment which may be useful]

Use these equipment if necessary:

- Microtube rack



- Plate rack



- Vortex mixer

- Centrifuge for microtube

- Centrifuge for test tube

- Float plate
  Use when the sample is heated with water bath.

- UV transilluminator
  Use when the sample is detected with Fluorescent detection solution.
  Transilluminator should output the light with wavelength of 240-260 nm or 350-370 nm

- UV goggles or face shield

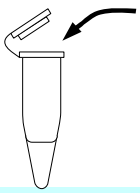
4. Instructions

[Simple protocol] Following is the simplified protocol described by way of example utilizing Hydrangea as specimen.

For the detailed protocol of this kit, please refer the instruction at page 7 and later.

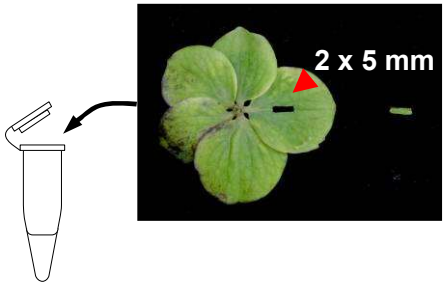
Simplified Protocol (In case of Hydrangea as specimen)

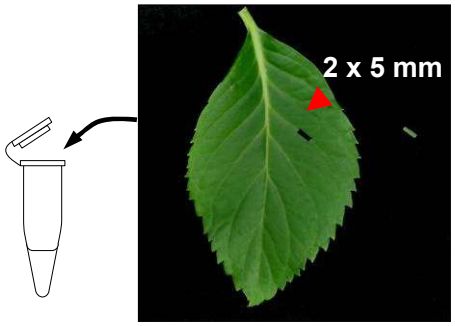
1. Dispense 100.0 µl Pytoplasma Extraction Solution to a sample tube (1 tube per sample)



Phytoplasma Extraction Solution
100.0 µl

2. Cut a small piece of Hydrangea calyx or leaf vein and soak it to 100 µl Phytoplasma Extraction Solution





3. Incubate the solution with sample piece at 95 ˚C for 10 minutes (this becomes DNA sample)

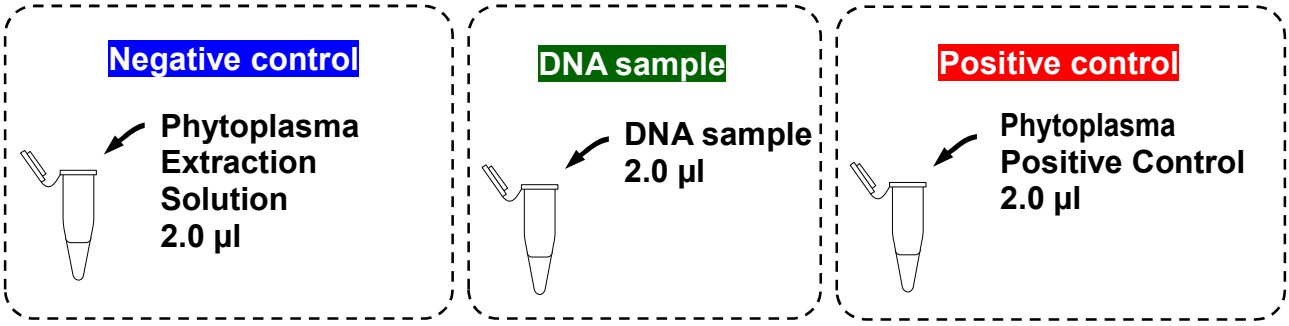
4. Prepare the test solution

Reagent	1test	8+1test*	48+1 test*
Phytoplasma Detection Solution	21.0 µl	189.0 µl	1029.0 µl
Fluorescent Detection Solution	1.0 µl	9.0 µl	49.0 µl
Phytoplasma Enzyme Solution	1.0 µl	9.0 µl	49.0 µl
Total	23.0 µl	207.0 µl	1127.0 µl

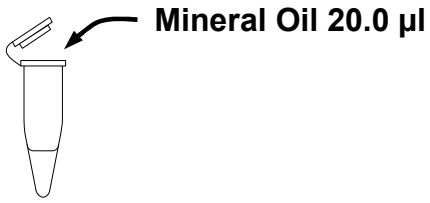
* Prepare the test solution for the number of the samples that you test + 1 test, so that you have enough volume for the test solution of all samples.

5. Aliquot 23.0 µl of the test solution to the test tube

6. Add 2.0 µl of DNA sample to the test solution



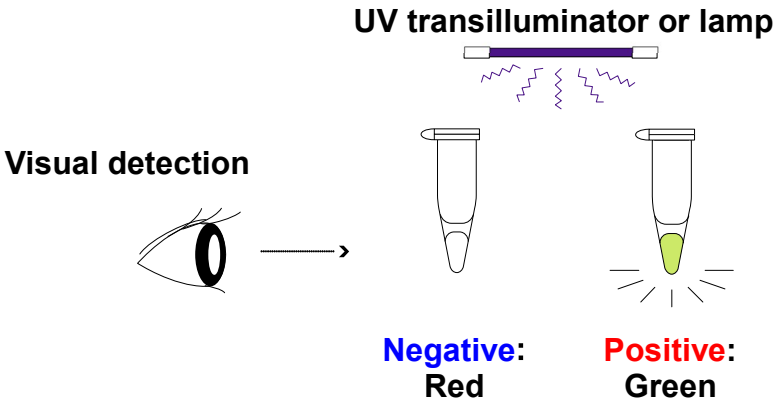
7. Add 20 µl of Mineral Oil

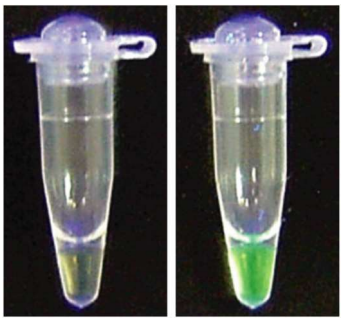


8. Incubate at 64˚C for 60 minutes (DNA amplification)

9. Incubate at 80˚C for 2 minutes (Enzyme inactivation)

10. Judge the result





[Preparation and precaution before detection]

Sample preparation

■ Control

This kit contains Phytoplasma Positive Control to confirm the success or failure of the detection results. To confirm the results are successful, attention should be taken to the preparation of “positive control reaction solution” with Phytoplasma Positive Control added, and also the “negative control reaction solution” with Phytoplasma Extraction Solution added.

■ Sample preparation

For detection of Phytoplasma, the kit utilizes DNA extracted from plant specimen. This kit contains one-step DNA extraction buffer (Phytoplasma Extraction Solution) to extract DNA from plant vascular bundle and a protocol for the extraction. Prior to detection, prepare the DNA with Phytoplasma Extraction Solution.

Important

If you use DNA extraction kit available on market or CTAB method to extract the DNA, keep the concentration of DNA sample 5 ng/μl or more.

Equipment preparation

■ Incubator

Turn on the power of incubator and set the required temperature. In case of using water bath and heat block, it might take time to reach the target temperature so pre-heat the device and confirm the temperature with thermometer. In case of using 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 air incubator.

■ Other equipment

equipment	Instruction
Micropipette	Monopolize micropipette for LAMP working area. Return to the original location after nucleic acid removal operation in case if used in other area.
Microtube rack	Monopolize microtube rack for LAMP working area. Return to the original location after nucleic acid removal operation in case if used in other area.
Microtube	Select gamma-ray sterile, nucleic acid-free, nuclease-free grade microtube.
Filtered pipette tip (sterilized)	Select gamma-ray sterile, nucleic acid-free, 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 a disposable 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 disposables, change them when the contamination is suspected.
White robe	Use solely in each working area, be noticed of the contamination from the cuffs.

Testing Environment

Because LAMP method is the DNA amplification technology with excellent sensitivities, it would be difficult to make an accurate inspection if the testing environment is contaminated with Phytoplasma positive control or amplified sample after test. For the handling of the sample, take extra care to avoid the contact of the positive control and samples to the working white lobe and equipment. It is also strongly encourage thorough exchanging of the clothing. 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 used for nucleic acid extraction and amplification (which has not been contaminated by nucleic acid) as dedicated reagent preparation area. Prepare the test solution only at the reagent preparation area. Do not use Phytoplasma 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 Phytoplasma 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 amount 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. Sodium hypochlorite generates chlorine gas and corrosive on metals, so 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 take attention of the expiratory date and storage temperature of the solution.

<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 paper towel moistened with sodium hypochlorite aqueous solution followed by wiping with 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 sample extraction (In case of Hydrangea phyllody Phytoplasma DNA)

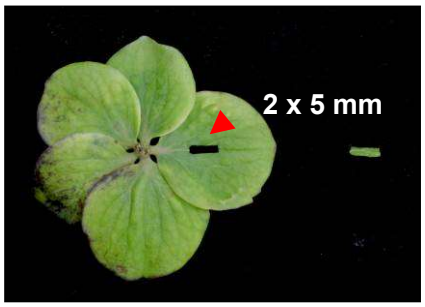
Important

This kit contains one-step DNA extraction buffer (Phytoplasma Extraction Solution) to extract Hydrangea phyllody Phytoplasma DNA from Hydrangea calyx or leaf vascular bundle and protocol for the extraction.

If you use commercial DNA extraction kit or CTAB method to extract the DNA, keep the concentration of DNA sample 5 ng/µl or more.

Thawing of the reagents

Completely Thaw the Phytoplasma Extraction Solution at room temperature. Keep the cap closed and invert to mix upside down 10 times until homogeneity. Dispense 100.0 µl to a microtube (1.5 or 2.0 mL, one tube per sample).

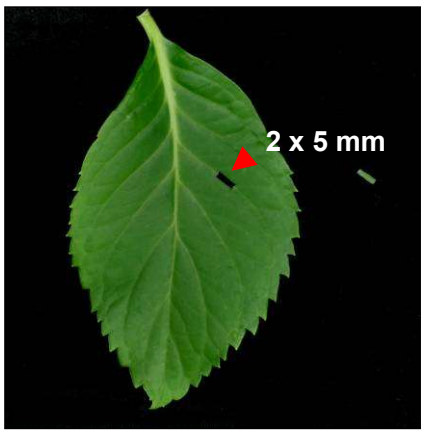


In case of calyx

Collection of plant vascular bundle sample

Collect the calyx or leaf from subjected Hydrangea trees, separately for each tree in a clean polyethylene bags. After collecting in the bags, handle the specimen with a tweezer. The tip of tweezer must be burned with alcohol lamp or burner or wiped with paper towel moistened with ethanol.

Use clean razor to cut the vascular bundle part of calyx or leaf into a size of 2 x 5 mm (figures left).



In case of leaf

DNA extraction

Soak completely the small piece of Hydrangea calyx or leaf vein in Phytoplasma extraction solution in the microtube. Incubate the microtube at 95˚C for 10 minutes. The solution obtained is the DNA solution. The solution is ready to use and it is not necessary to eliminate the Hydrangea piece from the solution or to vortex the solution.

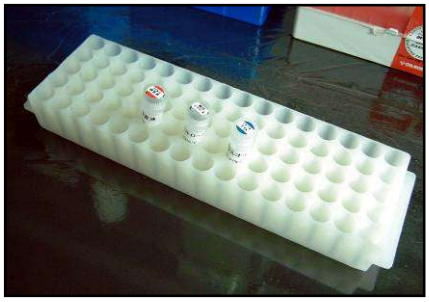
Important

Phytoplasma tends to localize at the phloem, so it is crucial to collect the part that contain vascular bundle.

Take an extra care of Phytoplasma Extraction Solution not getting contaminated; since it would be extremely difficult to make an accurate inspection once contaminated. In case of aliquot unused Phytoplasma Extraction Solution to microtubes and store, dispense 100 µl of the solution to each microtube and store at -20˚C under dark place.

Test reaction

Thawing of the reagents



Thaw the following reagents at room temperature.
Phytoplasma Detection Solution
Phytoplasma Positive Control
Mineral Oil
For Phytoplasma Enzyme Solution and Fluorescent Detection Solution, 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 at the bottom of the tubes (hereinafter referred to as “spin down”).

The reagents, Phytoplasma Enzyme Solution and Fluorescent Detection Solution, are placed on ice.

Preparation of Test Solution

To make Test Solution, add Phytoplasma Detection Solution, Fluorescent Detection Solution, and Phytoplasma Enzyme Solution to a sterilized microtube (1.5 ml or 2.0 ml). Mix the reagents by tapping or vibrating 3 times for approximately 1 second on a vortex mixer, and spin down the tube. Place the tube on ice.

Refer also to the following table.

[Table of volume]

Reagent	1 test	8+1 tests*	48+1 tests*
Phytoplasma Detection Solution	21.0 µl	189.0 µl	1029.0 µl
Fluorescent Detection Solution	1.0 µl	9.0 µl	49.0 µl
Phytoplasma Enzyme Solution	1.0 µl	9.0 µl	49.0 µl
Test Solution Total	23.0 µl	207.0 µl	1127.0 µl

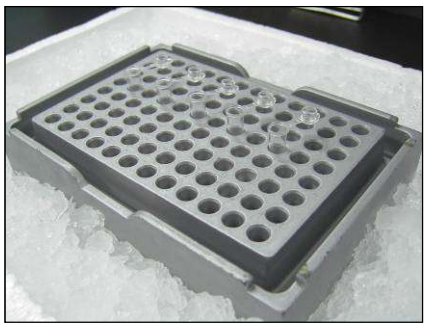
* Prepare the test solution for the number of the samples that you test + 1 test, so that you have enough volume for the test solution of all samples.

Important

Consecutive dispensing of the reagent may cause cross-contamination, use the filtered tip as a disposable in every dispensing batch.

Phytoplasma Enzyme Solution is extremely viscous so be careful not to stick too much amount of solution on the outer surface of the filtered micro tip. Spin down prior to use.

Distribution of Test Solution



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

Important

Only use the specified Test Tube which is attached to this kit. Use of different volume, shape, and material may cause misjudgment.

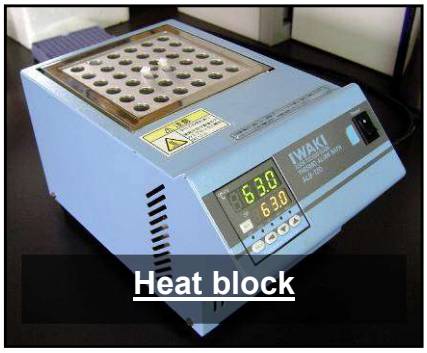
DNA sample addition

Add 2.0 µl DNA sample to the Test Tube. Then add 20 µl of Mineral Oil to prevent evaporation of a reagent and close the cap. (Be noticed that the test reaction efficiency drastically decrease if the test solution gets concentrated). If a thermal cycler having the function of hot bonnet heated lid is used as incubator, mineral oil addition is not necessary.

Important

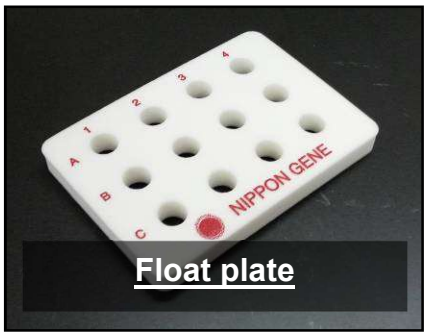
When adding the DNA sample and Mineral Oil, be sure to operate with the following order.
1: Negative Control Sample
2: DNA sample
3: Positive Control Sample
Close the cap immediately after sample addition.
Further, add 2.0 µl Phytoplasma Extraction Solution and Mineral Oil to a Negative Control sample and use 2.0 µl Phytoplasma Positive Control for a positive control sample.

Test Reaction



Heat block

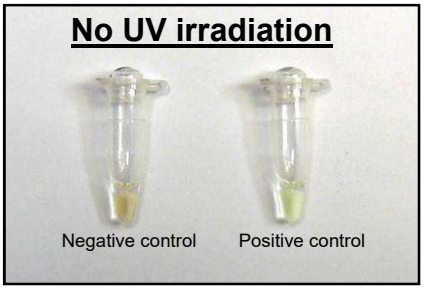
Close Test Tube and mix the reagents by tapping or vibrating on a vortex mixer at about 1 second x 3 times, and spin down the tube. Incubate Test Tube at 64˚C for 60 minutes in the incubator (Air incubator, heating block, or water bath).
In case of using water bath, use float plate to prevent the Test Tube tilting horizontally.

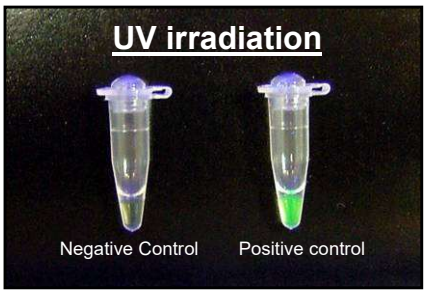


Float plate

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 before reaction looks light red. If Phytoplasma exists, it turns to vivid green under UV irradiation.
In this procedure, UV transilluminator (wavelength: 254 - 366 nm), UV goggles, or a face shield is needed.
Normally, Positive Control Solution appears fluorescence and Negative Control Solution does not appear fluorescence. If Negative Control Solution appears fluorescence, there is possibility that Phytoplasma Positive Control enters the test tube by mistake. In this case, check the handling and try to test again.

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

Judgement of the sample

After judgement of the control solutions having done and the test is judged as valid, do the judgement of the results of sample. If Test Solution emits the fluorescence that is same level with the Positive Control Solution under UV irradiation, the sample may contain Phytoplasma. If the fluorescence is NOT detected under UV irradiation, the sample does not contain Phytoplasma.

[Tips of judgement]

Test Solution emits fluorescence clearly

The plant may be infected with Phytoplasma. Even if the emission is weak, when difference can be observed between negative control solution and sample solution, there might be infection. In such case, collect other sample from different part of the subjected plant and redo the test.

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

The plant may not be infected with Phytoplasma. However, fluorescence may not appear because of uneven distribution of Phytoplasma in plant body. Observe well the presence of disease symptom and if infection is suspected, collect several sample from different part of the subjected plant and redo the test.

Important

If Phytoplasma infection is suspected regardless to the results given by this kit, please consult with The University of Tokyo, Plant Clinic.

The University of Tokyo, Plant Clinic;

http://park.itc.u-tokyo.ac.jp/ae-b/hospital/

Place: **Yayoi 1-1-1, Bunkyo-ku, Tokyo**
TEL: **03-5841-0567**
E-mail: **byoin@todaiagri.jp**

5. Troubleshooting

If you experience trouble with this kit, check the items on 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 Phytoplasma Positive Control added to the test solution** There are some cases that efficiency of test reaction decreases when too much Phytoplasma Positive Control is added to the reaction. Please follow the instruction for the correct amount of addition. **B. Reagents or testing environment are contaminated with nucleic acid** In case of negative control testing solution gives coloring, template DNA contamination is suspected. Contamination monitoring of reagents and testing environment, cleaning procedure by 1% sodium hypochloride aqueous solution are recommended to remove completely the contaminants. After the removal, redo the test. **C. Chelete compounds or metal ion in the sample** The Fluorescent Detection Solution emits fluorescence when chelate compounds such as EDTA exists in the reaction. On the other hands, if a lot of metal ion presents in reaction, the fluorescence is inhibited thus it would be difficult to judge the result. **D. Reaction temperature and operating procedure not correct** Confirm that there is no problem on the test process.
Irregular coloring of fluorescent detection solution	**A. Judgement not immediately after test reaction has ended** Fluorescent Detection Solution irregularly gains or loses its coloring when left in room temperature for long time. Follow the instruction for storage and handling of the solution.
Test solution has evaporated.	**A. The reaction tube not heated homogeneously** Water bath, heat block may have not heated the test tube homogeneously so that the test solution would be concentrated because of evaporation. In such case the reactivity efficiency goes down. Make sure that mineral oil to be added to the test solution.
The judgement of fluorescence is difficult.	**A. UV lamp wavelength 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).
There are not enough reagents for testing.	**A. Reagent sticks on the inner tube surface.** Spin down the microtube before use. **B. Reagent evaporated during its storage** Completely close the cap after use.

6. Reference

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7. Supplementary data

[Quality control]

It has been confirmed that the emission of fluorescence was detected when DNA amplification reaction was done with attached Fluorescent Detection Solution, and 2.0 µl of Phytoplasma Positive Control as template in 25.0 µl reaction volume under 64˚C temperature for 60 minutes.

[Copy number of Phytoplasma Positive Control]

Phytoplasma Positive Control contains 10^7 copies of target sequence per 2.0 µl volume.

[Memo]

[Memo]

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Information

NIPPON GENE CO., LTD.

Address 2-7-18, Toiya-machi, Toyama 930-0834
Japan
TEL +81-76-451-6548 FAX +81-76-451-6547
URL http://nippongene-analysis.com
E-mail support@nippongene-analysis.com