DrACDD™

Phytoplasma Universal Detection LAMP Kit

Instruction Manual

version 3. 0. 0

Nippon Gene Material

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[Read the following instructions before the test]

Thank you very much for purchasing **DryADD™ Phytoplasma Universal Detection LAMP Kit**. Before using the kit, please confirm the following matters.

Notices for use

1. 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.

2. Concerning the storage procedure of the kit, please read section 2 “Notes” for your reference. The unopened kit is stable at room temperature (20-25˚C). Use within the expiration date.

3. Use this kit according to this instruction manual. Nippon Gene Material 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 Material 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 in order 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 7).

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

1.About the kit

Product Overview

DryADD™ Phytoplasma Universal Detection LAMP 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 test is extremely easy, simply to mix the DNA extract from plant sample with the test solution (dissolved Phytoplasma Detection Dry Reagent), and keep the solution at 64 degrees 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, so 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.

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

Eiken GENOME SITE; https://loopamp.eiken.co.jp/en/

2. Reagents provided with the kit

[Kit components (for 48 tests)]

Reagent	Form (top label)	contents (48 tests)	Storage temperature
Instruction Manual	Booklet	1 booklet	-
Phytoplasma Detection Dry Reagent	Aluminum bag	8 well x 6 strips	Room temperature
Phytoplasma Reagent Dissolve Solution	Orange	400 µl x 6 tubes	
Phytoplasma Extraction Solution	White	1 ml x 48 tubes	
Phytoplasma Positive Control	Gray	6 tubes	
Phytoplasma Control Dissolve Solution	Green	400 µl x 1 tubes	
Phytoplasma Negative Control	Light Blue	400 µl x 6 tubes	
Mineral Oil	Blue	400 µl x 6 tubes	

Notes

- Store all reagents in the aluminum bag at room temperature (20-25˚C). Protect them from light. Use within the expiration date.
  Especially, Phytoplasma Detection Dry Reagent and Phytoplasma Positive Control should be kept in aluminum bag with desiccant agent to prevent degradation by humidity.

- This kit can perform 6 test reactions when you use 8 well per 1 test. To prevent misjudging derive from contamination of DNA, it is recommended to dispose Phytoplasma Reagent Dissolve Solution, Phytoplasma Positive Control, Phytoplasma Negative Control, Mineral Oil after each test.

- Phytoplasma Detection Dry Reagent is dispensed in 8 well strip tube. Please adjust the tube number by cutting the strip with scissors depending on your test size.

- When storing dissolved Phytoplasma Positive Control or DNA sample after extraction, please keep it separate from other reagents.

- To prevent misjudging, do not keep dissolved Phytoplasma Detection Dry Reagent at room temperature or in a refrigerator for a long time, or freeze it by excessively cold condition.

- 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.

3.Instructions

Here describes an example of phytoplasma detection from Coconut palm as a specimen.

To avoid template DNA contamination, please separate **"template DNA preparation (step A, B) room"** and **"reagent preparation (step C) room"** for testing.

Simplified Protocol
(In case of Coconut palm as specimen)

* Please do not proceed all procedure at the same time,
  carry out each process in order of A → B → C.
* Please wear disposable gloves during step A to C and change them when you go next step.

<A. preparation of DNA sample>

A-1. Take out the required number of **Phytoplasma Extraction Solution** tube.
* Before opening the lid, spin down the tube.

A-2. Scale 0.005 g of Coconut wood chip from stem and soak it completely in **Phytoplasma Extraction Solution**.
* Use a drill to collect chip from stem of *Cocos nucifera*.

A-3. Invert to mix upside down, then incubate the microtube at 95˚C for 10 minutes.
* Incubate the tube after chip and **Phytoplasma Extraction Solution** are mixed completely.

A-4. Invert to mix upside down, then cool with water to room temperature (the solution obtained is **DNA sample**).
* After 10 minutes incubation, make sure to invert and homogenize **DNA sample**.
* Make sure to cool the **DNA sample** before adding it to **test solution**.

<B. Preparation of Phytoplasma Positive Control Solution>

B-1. Take out one of **Phytoplasma Positive Control** tube.
* To avoid absorption of moisture, the remaining tubes should be sealed in aluminum bag immediately.

B-2. B-1. Add 10 µl of **Phytoplasma Control Dissolve Solution**.

B-3. After spinning down, leave it at room temperature for 5 minutes.
* Because the dried **Phytoplasma Positive Control** exists in the bottom of tube, make sure to contact the control with **Phytoplasma Control Dissolve Solution** completely.

B-4. After vortexing, spin down the tubes (**Phytoplasma Positive Control Solution**).

<C. Preparation of test solution and LAMP reaction>

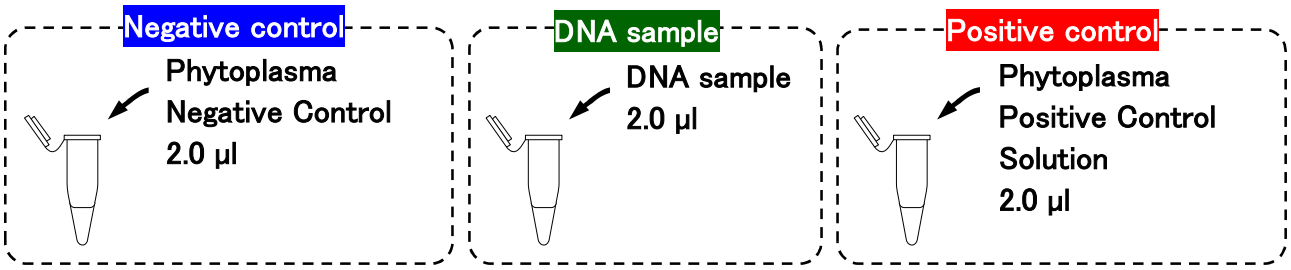
C-1. Take out the required number of **Phytoplasma Detection Dry Reagent**.

* The tubes of required number (sample number and control number) must be kept on ice (using aluminum rack or plate rack) .
* To avoid absorption of moisture, the remaining tubes should be sealed in aluminum bag immediately.
* If the dry reagent is attached on lid, shake the tube gently and pull down the reagent at bottom of tube.

C-2. Add 23 µl of **Phytoplasma Reagent Dissolve Solution** to each tube.

C-3. Add 2 µl of sample, close the cap, and spin down.

* When adding the sample, be sure to operate with the following order:
  1. Negative control, 2. DNA sample, 3. Positive control
* **DNA sample** contains small wood chip on top of the solution, and big wood chip on bottom.
  Take **DNA sample** without wood chips from the middle portion of the solution.
* After **Phytoplasma Detection Dry Reagent** dissolved, small bubble will occur,but this will not affect the testing reaction.
  To eliminate the bubble, let the tube stand for 2 minutes, invert 5 times to mix, then spin down.



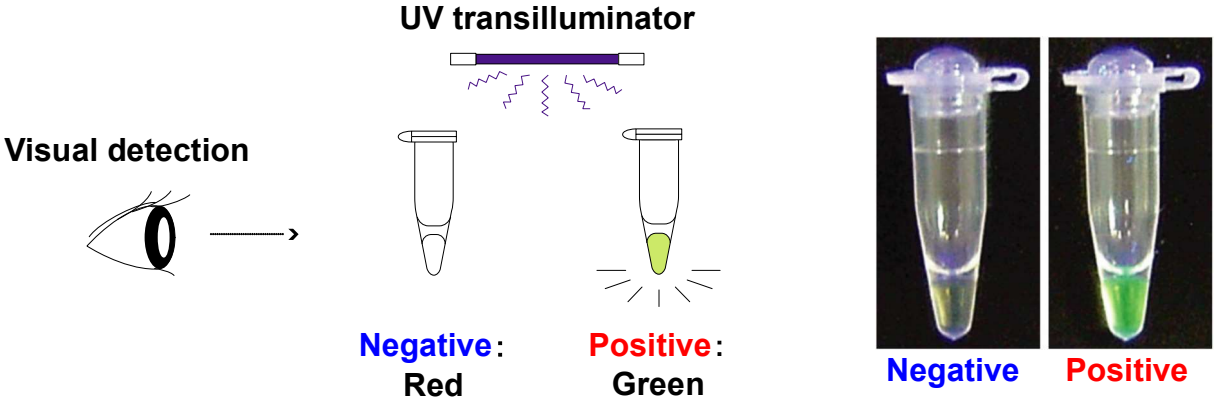
C-4. After 2 minutes, overlay with 20 µl of **Mineral Oil**.

* If dry reagent and **Mineral Oil** come into contact, it may cause insoluble precipitation.
  If you use **Mineral Oil**, make sure to add it after dry reagent are completely dissolved.

C-5. Incubate test tube at 64˚C for 60 minutes (test reaction).

C-6. Terminate the reaction at 80˚C for 2 minutes.

C-7. Judgement



4. Troubleshooting

If you experience trouble with this kit, check the items on below and try the solutions. Consult Nippon Gene Material 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 hypochlorite aqueous solution are recommended to remove completely the contaminants. After the removal, redo the test. **C. Chelate compounds or metal ion in the sample** Test 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 test solution	**A. Judgement not immediately after test reaction has ended** Test Solution irregularly gains or loses its coloring when left at room temperature for long time. Please judge immediately after LAMP reaction finished.
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.

5. Reference

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