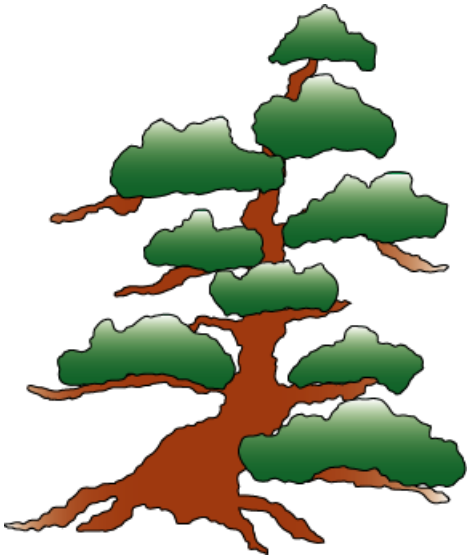
Bursaphelenchus xylophilus Detection Kit

Instruction Manual

Version 2.0



NIPPON GENE CO., LTD.

Bursaphelenchus xylophilus Detection Kit

Instruction Manual Version 2.0

[Read the following instructions before the test]

Before use the kit, please confirm the following.

Notices for use

- This kit is the product to detect *Bursaphlenchus xylophilus*. This kit must not be used for clinical diagnosis, therapeutic purpose and the test other than *Bursaphlenchus xylophilus* detection.
- Use the 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.
- The unopened kit is stable at -20˚C for 6 months. It should be kept in dark. Avoid repeated freezing and thawing.
- Forestry and Forest Products Research Institute is applying for a patent of *Bursaphlenchus xylophilus* detection using LAMP method. Nippon Gene Co., Ltd. has been granted the license to detect for *Bursaphlenchus xylophilus* using LAMP method.

  Refer to the Safety Data Sheet (SDS) about safe use of this product. Please inquire through the inquiry form you can find in the URL.
- 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.

Table of contents

1. About the kit

[Product overview]

This kit is the product to detect *Bursaphlenchus xylophilus* by isothermal amplification reaction using LAMP (Loop-mediated Isothermal Amplification) method. This product can test whether *Bursaphlenchus xylophilus* exists in pine wood chips. In this kit, a part of *Bursaphlenchus xylophilus* DNA is amplified using LAMP method, and *Bursaphlenchus xylophilus* infection is judged by the existence of amplification. This kit is effective for the highly-sensitive detection of pine wilt disease.

[About LAMP method]

LAMP (Loop-mediated isothermal amplification) method allows the whole reaction process, including denaturating, proceeds at a constant temperature in an incubator. Thermal cycling machine is not needed for this kit.
Look at 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 24 or 96 tests)]

Form	Reagent	Contents		Storage temperature
		24 tests	96 tests	
Booklet	**Instruction Manual**	**1 sheet**	**1 sheet**	Room temperature
Red label	**Bx Detection Solution**	**410 µl**	**4x 410 µl**	-20˚C
Yellow label	**Bx Enzyme Solution**	**20 µl**	**4x 20 µl**	-20˚C
Purple label	**Fluorescent Detection Solution**	**20 µl**	**4x 20 µl**	-20˚C
Gray label	**Bx Positive Control**	**25 µl**	**4x 25 µl**	-20˚C
Blue label	**Mineral Oil**	**500 µl**	**4x 500 µl**	-20˚C
Bottle	**Bx Extraction Solution**	**2x 10 ml**	**8x 10 ml**	-20˚C
Bag	**Extraction Tube**	**24 tubes**	**100 tubes**	Room temperature
Bag	**Test Tube**	**24 tubes**	**4x 24 tubes**	Room temperature

Notes

Store all reagents other than **Instruction Manual** and **Extraction Tube**, **Test Tube** at -20˚C. Avoid storage in extremely cold environments, and avoid repeated freezing and thawing.
This kit can be used for 6 months after purchase.
Enzyme Solution must be handled with great care. Do not leave it at room temperatures or 4˚C for a long period of time, or the enzyme may lose activity.
Bx Positive Control is the DNA that contains a fragment of *Bursaphlenchus xylophilus* genomic DNA.
When waterdrop is attached to the **Test Tube**, dry a bag well before opening.

Positive Control

Always run positive control with the sample. To prepare Positive Control Solution, add the provided **Bx Positive Control** (Gray label) instead of the sample.

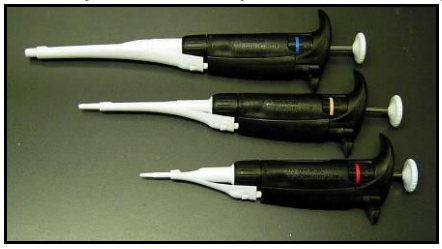
Negative Control

Always run negative control with the sample. To prepare Negative Control Solution, add nothing instead of the sample.

3. Equipments and reagents not provided with the kit

15 mm drill

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 glove

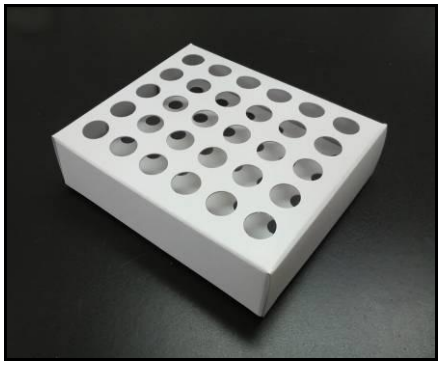
Incubator

Tweezers

Crushed ice

Plastic bag

Microtube rack

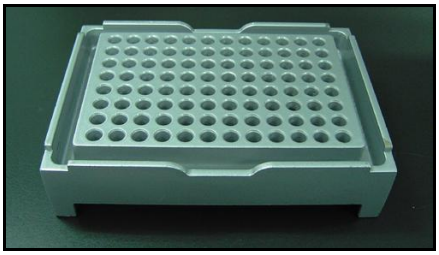


Vortex mixer

Centrifuge for microtube

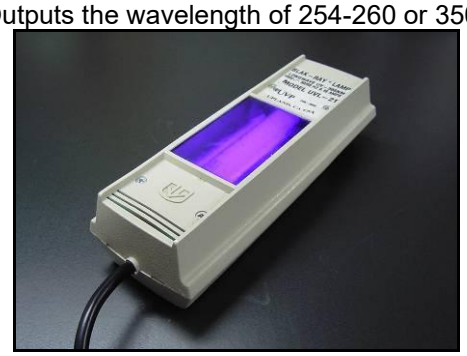
Centrifuge for test tube

Plate rack



UV transilluminator

Outputs the wavelength of 254-260 or 350-370 nm



UV goggles or face shield

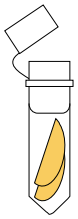
4. Instructions

[Simple protocol]

Simple protocol

1. Collect pine wood chips with 15 mm drill

2. Two chips are put in 800 µl of Bx Extraction Solution



Bx Extraction Solution

3. Keep warm for 20 minutes by 55˚C

4. Heat with 94-100˚C for 10 minutes (DNA Sample)

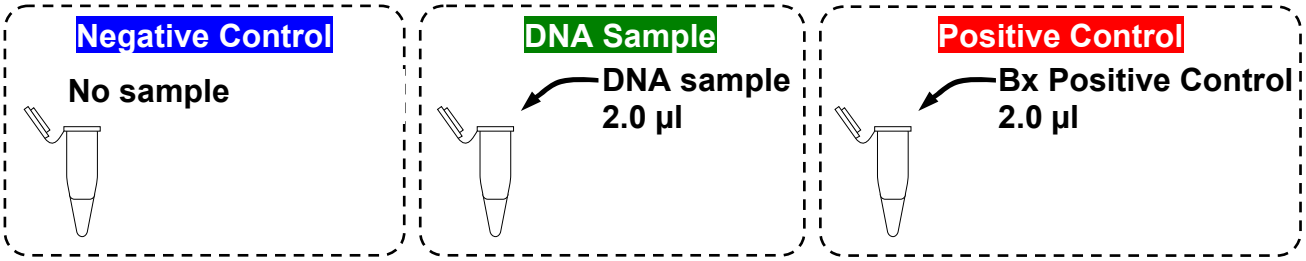
5. Prepare Test Solution

Reagent	1 test	8 test	24 test
Bx Detection Solution	16.4 µl	131.2 µl	393.6 µl
Fluorescent Detection Solution	0.8 µl	6.4 µl	19.2 µl
Bx Enzyme Solution	0.8 µl	6.4 µl	19.2 µl
Total	18.0 µl	144.0 µl	432.0 µl

Simple protocol

5. Divide Test Solution into each Test Tube (18.0 µl per tube)

6. DNA sample prepared in process 4 is added 2.0 µl each Test Tube



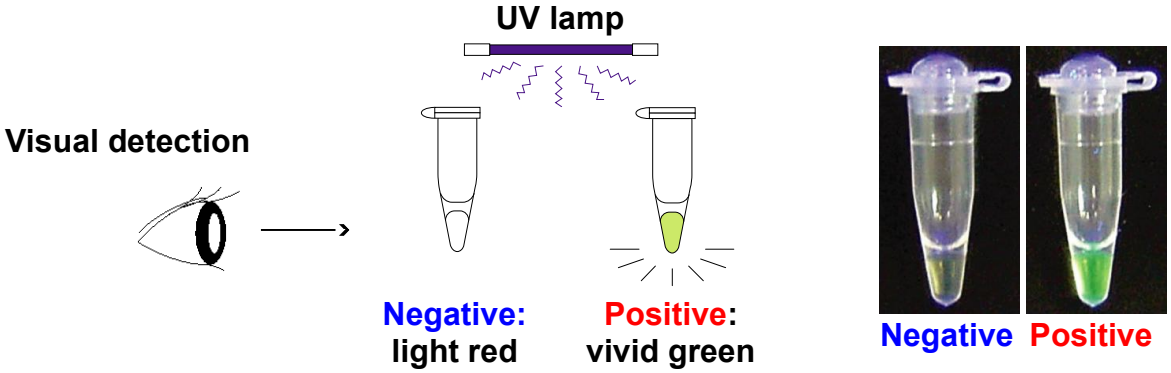
7. Add Mineral Oil

6. 63˚C, 60 minutes (DNA amplification)

* Basically, incubation is set to 60 minutes.
But, when using the pine wood chips have remarkably low density of the nematode, the detection rate sometimes rises by extending it until 90 minutes.

7. 80˚C, 2 minutes (Enzyme inactivation)

8. Judge the result



Bursaphelenchus xylophillus detection

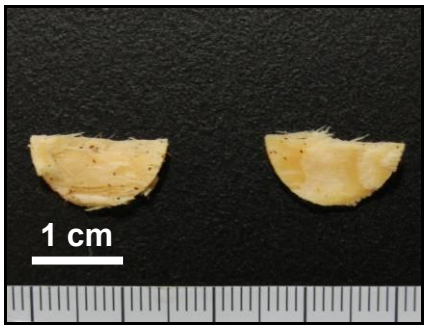
DNA sample preparation

Collection of pine wood chips

Pine wood chips are collected from a pine tree using a 15 mm drill. You should extract a pine wood chips from more than one points every height of the tree to check it more correctly.

DNA extraction

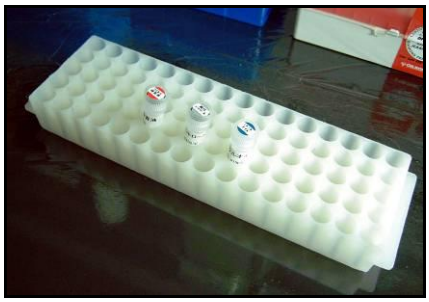
Thaw **Bx Extraction Solution** at room temperature. The bottle is mixed by inversion.
800 µl **Bx Extraction Solution** is added to **Bx Extraction Tube**.
Next 2 chips are put in **Bx Extraction Solution**.
After keeping a tube for 20 minutes by 55˚C, it's kept warm for 10 minutes by 94-100˚C, and the obtained solution is called a DNA sample.



Important
When more than 3 pine wood chips are used, the efficiency falls in extraction and after test.

Test reaction

Thawing of the reagents

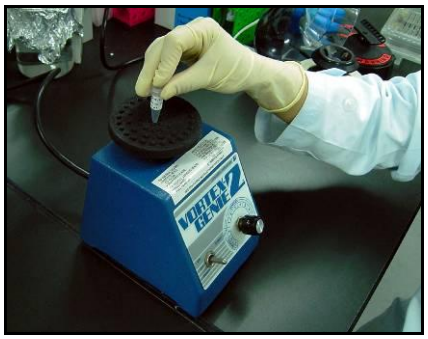


Thaw the following reagents at room temperature.

Bx Detection Solution
Bx Positive Control
Mineral Oil

Bx Enzyme Solution and **Fluorescent Detection Solution** do not freeze at -20˚C.

Mix and spin down



Gently tap the tubes several times (This operation is called 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 (this operation is called spin down).
The reagents, **Bx Enzyme Solution** and **Fluorescent Detection Solution**, are placed on ice.

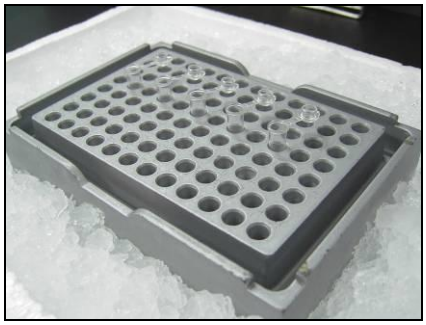
Preparation of Test Solution

To make Test Solution, add **Bx Detection Solution**, **Fluorescent Detection Solution**, and **Bx 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. It places on ice.
Refer also to the following table.

[Table of volume]

Reagent	1 test	8 tests
Bx Detection Solution	16.4 µl	131.2 µl
Fluorescent Detection Solution	0.8 µl	6.4 µl
Bx Enzyme Solution	0.8 µl	6.4 µl
Total	18.0 µl	144.0 µl

Distribution of Test Solution



Pick a tube out with clean tweezers, etc. from a bag. Divide 18.0 µl of Test Solution into each **Test Tube**.

DNA sample addition

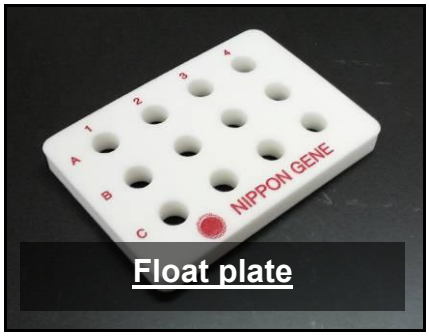
2.0 µl DNA sample will be added to **Test Tube**. Then 20 µl of **Mineral Oil** is added to prevent evaporation of a reagent.

Important

When add 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 a cap immediately after sample addition.
Further, add only **Mineral Oil** to a Negative Control sample and use 2.0 µl **Bx Positive Control** for a positive control sample.

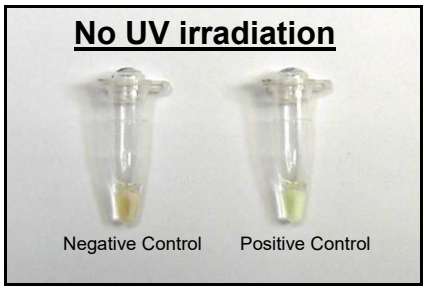
Incubation

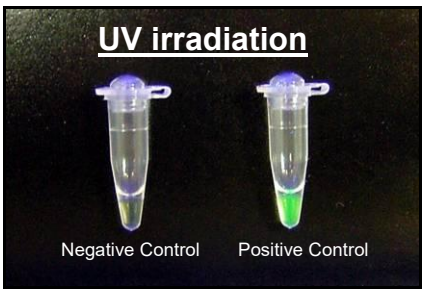
Close Test Tube and mix the reagents by tapping or vibrating on a vortex mixer at about 1 second x 3 times, and spin down the tube.
Incubate Test Tube at 63˚C for 60 minutes in the incubator (Air incubator, heating block, or water bath).



Judgment

Judgment of success or failure of the test





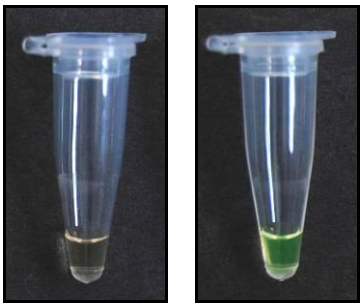
After 60 minutes passes, terminate the reaction by heating for 2 minutes at 80˚C.
Fluorescence Detection Solution before reaction looks light red. If *Bursaphelenchus xylophilus* exists, it turns to vivid yellow under UV irradiation.
In this procedure, UV transilluminator (wavelength: 254 - 366 nm), 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 **Bx Positive Control** enters the test tube by mistake. In this case, check the handling and try to test again.

Important
Judge the result immediately after the reaction is completed.

Important

Use of UV irradiation equipment is being recommended as judgment by this kit.
But, when coloring is strong, when a tube is put on black paper, it's possible to confirm the coloring by a naked eye.

No UV irradiation
On the black paper



Negative Control　　Positive Control

Judgment of the sample

[Judgment]

If Test Solution appears the fluorescence that is same level with the **Positive Control Solution** under UV irradiation, the sample may contain *Bursaphelenchus xylophilus*. If the fluorescence is NOT detected under UV irradiation, the sample does not contain *Bursaphelenchus xylophilus*.

[The tips of judgment]

Test Solution has emitted fluorescence clearly

The plant may be infected with *Bursaphelenchus xylophilus*.

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

The plant may not be infected with *Bursaphelenchus xylophilus*. However, fluorescence may not appear in the early stage of infection, since nematode concentration is very low.

6. References

1. Aikawa T, Kikuchi T, Kosaka H. (2003) Demonstration of interbreeding between virulent and avirulent populations of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) by PCR-RFLP method. *Appl. Entomol. Zool.* **38** (4): 565

2. Kikuchi T, Aikawa T, Oeda Y, Karim N, Kanzaki N. (2009) A rapid and precise diagnostic method for detecting the Pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. *Phytopahology*. **99** (12): 1365

3. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. **28** (12): e63

4. Prince AM, Andrus L. (1992) PCR: how to kill unwanted DNA. *Biotechniques.* **12** (3): 358

5. Kanetani S, Kikuchi T, Akiba M, Nakamura K, Ikegame H, Tetsuka K (2011) Detection of Bursaphelenchus xylophilus from old discs of dead Pinus armandii var. amamiana trees using a new detection kit. *Forest Pathology* **41** (5): 387

6. Hoshizaki K, Nakabayashi Y, Mamiya Y, Matsushita M (2016) Localized within- and between-tree variation in nematode distribution during latent state of pine wilt disease makes the disease status cryptic. *Forest Pathology* **46** (3): 200

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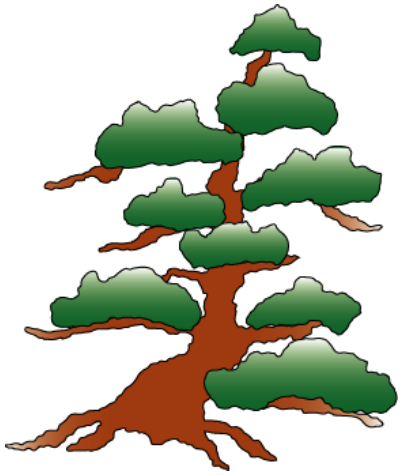

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