

# **PCR Kit for the detection of *Erwinia amylovora* (Fire blight)**

## **Instruction Manual**

Microsynth AG  
Schützenstrasse 15  
9436 Balgach  
Switzerland

Phone ++41 71 722 8333  
Fax ++ 41 71 722 8758  
[administration@microsynth.ch](mailto:administration@microsynth.ch)  
[www.microsynth.ch](http://www.microsynth.ch)



## PCR Kit for the detection of *Erwinia amylovora* (Fire blight)

### Content

	96 PCR tube – Kit Article No. 1000-96	384 PCR tube – Kit Article No. 1000-384	1536 PCR tube – Kit Article No. 1000-1536
DNA Extraction Buffer	1 x 100ml	1 x 400ml	4 x 400ml
PCR tubes	96 tubes	384 tubes	1536 tubes
dNTP's 25mM each, 22µl	1 tube	4 tubes	16 tubes
Positive Control, 300 µl	2 tubes	2 tubes	6 tubes
1 Manual			

The PCR tubes contain a lyophilized mixture of primers, PCR salts and internal control DNA.

### Shipment and Storage Temperature

Shipment: Room Temperature. Rigorous tests at Microsynth have shown that a shipment time over at least 10 days at room temperature does not influence the quality of this kit.

Storage: PCR Tubes, dNTP's and Positive Control at -15 to -25 °C  
DNA Extraction Buffer at 2 - 8 °C

### Stability

At least 1.5 years at above storage temperature

### Equipment needed

Heating Block  
Pipetting device and tips with integrated filters  
1.5ml or 2ml screw cap plastic tubes  
Agarose gel electrophoresis equipment  
Centrifuge  
PCR machine  
Hot Start PCR Polymerase

### Preface

Microsynth recommends to follow the general rules to prevent PCR cross contamination. Although we have observed that cross contamination is no problem with this kit, tips with integrated filters should be used for all pipetting work. The following three steps are usually performed in three separated rooms:

- Extraction room: Cutting plants and addition of Extraction Buffer, heating and dilution
- PCR-buffer room: Addition of Mastermix and DNA-Extract to the PCR tubes\*
- Gel electrophoresis room: PCR machines and gelelectrophoresis. Do not open the PCR tubes after the PCR reaction in the PCR buffer room!

\*It is a good idea to add the Mastermix in a Laminar Flow (if available) and the DNA-Extract outside of the Laminar Flow.

## Principle

This kit is based on a nested PCR reaction from plasmid pEA29 published by Maria M. Lopez, Instituto Valenciano de Investigaciones Agrarias, 46113 Moncada, Spain and has been improved by Microsynth. Main advantages are:

Speed (the detection of fire blight is possible within 6 hours after the beginning)

No precultivation of *E. amylovora* bacteria – direct PCR from stems, leaves, fruits or blossoms.

The PCR reaction performs 67 cycles, this special high amplification exceeds by far the normal PCR procedures using just 35 cycles and results in a very high sensitivity, even with a very low starting amount of DNA. Therefore, this method is unaffected by the presence of PCR inhibitors.

Sensitivity: 1 culturable *E. amylovora* cell is detectable with this PCR kit

High standardization. Each production lot is checked at Microsynth with our positive control and has to meet the quality standards mentioned on the supplied data sheet. Therefore, a lot-to-lot variation is excluded. The only source of variation is the way how the sample is taken from the plant.

## Pework

Dissolve the powder of the DNA Extraction Buffer in sterile water. The appropriate volume is indicated on the bottle. Let stand for 5 mins. to dissolve the powder and shake.

Before opening the nucleotides, centrifuge tube 10 secs. to ensure that no liquid is in the lid. Add 198 µl sterile water to the 22 µl nucleotides (25mM each nucleotide). This step will dilute the nucleotides to 2.5mM each.

## Short Description of this PCR – Kit

Small 1mm - pieces from infected blossom, buds, shoots or stems are transferred into a 1.5ml or 2ml – plastic vial with screw cap. Add 100 – 1000 µl of DNA Extraction Buffer. The small infected pieces should be immersed with buffer. Heat 20 mins. at 95 °C to extract *E. amylovora* DNA.

In the meantime, add 24 µl of a mastermix containing water, dNTP's and PCR polymerase to Microsynth's prefilled PCR tube.

Centrifuge the DNA extract for 10 seconds and dilute 1 µl supernatant in sterile water.

Add 1 µl diluted DNA extract to above 24 µl PCR reaction

Start the 3 hour – PCR reaction

Load 5 µl on a 1.5% agarose gel and run 30 mins.

Interpretation: The lower 139bp fragment shows the internal control. This band should be visible always. If this band is absent, a diagnostic interpretation is not possible. The upper 383 - 399bp fragment indicates the presence of *E. amylovora*.

## Detailed Description of this PCR – Kit

### a) Sampling

Small suspicious plant pieces are transferred into a 1.5ml or 2ml – plastic vial with screw cap. Careful working is very important. Small pieces from a non-infected part as a negative control should be tested as well. The age of the plant material is not important for this PCR-assay. At Microsynth, we received the same clear PCR result from a 5 months old stem which was completely dried. However, it is easier to localize bacteria in fresh wood.

### b) Extraction of *E. amylovora* DNA

Add 100 – 1000 µl of the supplied DNA Extraction Buffer to the tube containing the sample. This amount is not critical, but the piece(s) should be immersed with buffer. In the case that the buffer turns viscous during heating, simply dilute with more buffer. Put this tube on a heating block for 20 mins. at 95 °C and shake after 10 and after 20mins. The solution will be colorless, yellow or brown. Bacterial DNA will be extracted if present. After this extraction, centrifuge plant particles down (20 seconds at 10 – 17krpm). Take 1 µl of the supernatant and dilute in sterile water. Depending on the kind of material (Washed blossom with few bacteria, stem with many bacteria) a dilution of 10fold, 100fold, 1'000 fold or 10'000 fold should be made. This dilution was part of a thorough investigation at Microsynth. For stem, we have found optimal results with a 1'000 fold dilution. Undiluted extracts will contain PCR inhibitors and too much salts from the extraction buffer, therefore the extract must be diluted with water at a minimum of 10 fold.

### c) PCR amplification

The delivered PCR tubes contain salts, primers and a control DNA in a lyophilized form. The purpose of the control DNA is to indicate successful PCR conditions respectively absence of PCR inhibition. Nucleotides are delivered in a separate tube. Taq-Polymerase has to be supplied by the customer. Microsynth has tested several Taq-Polymerase enzymes and not all of them give optimal results for this test system. We strongly recommend to use only the tested enzymes mentioned in the table below!

Prepare a mastermix containing water, dNTP's and PCR polymerase. The volume of the mastermix should be calculated according to the number of the samples including the positive and negative control.

Mastermix for 1 tube:

21.7 µl sterile water  
2 µl diluted Nucleotides (2.5mM each nucleotide)  
0.3 µl Taq-Polymerase 5 Units per µl

Add 24 µl of this mastermix to each PCR tube, vortex and add 1 µl sample extract or 1 µl control for a final PCR volume of 25 µl. Mix gently and spin down if some liquid is in the lid.

Cycle conditions:

Step 1	94 °C	12 mins
Step 2	94 °C	30 secs
Step 3	72 °C	60 secs
Step 4	Goto step 2, 25x	
Step 5	94 °C	30 secs
Step 6	56 °C	30 secs
Step 7	72 °C	45 secs
Step 8	Goto step 5, 40x	
Step 9	15 °C	for ever
Step 10	End	

(Total time about 3 hours)

### d) Agarose gelelectrophoresis

During the PCR amplification, pour a 1.5% gel containing Ethidium bromide or similar intercalating dye. In the case that you need several gels per week / per month, we recommend to pour a larger gel and cut only the size of the gel you need. The non-used gel might be easily stored for weeks in the dark wrapped in a foil at room temperature. The two PCR fragments are short, therefore a separation distance of 45 mm is far enough. Load 5

µl of the PCR reaction on the gel and run until Bromphenolblue marker is migrated about 26 mm from the slot (normally 30 mins. with 200 Volts, 140mA). Both PCR bands will be around the Bromphenolblue dye. Tip: It is no problem to use the electrophoresis buffer up to 5 times before changing.

e) Interpretation of the result

The positive control will show a strong band of *E. amylovora* DNA at 399bp and a weaker band at 139bp (internal control).

The negative control will show the 139bp fragment only. If the upper fragment is visible, a cross contamination did occur. In this case, you should not interpret the PCR reactions done at the same time.

Your collected samples will always show the 139bp fragment. If this band is not visible, PCR inhibition occurred. In this case, never interpret your result! Repeat the PCR by further diluting the DNA extract. However, a 1 : 1000 fold dilution will work.

8bp – variations from world-wide fire blight have been observed. The size of your fragment might vary in your case.

If the 381 - 399bp fragment is present, your sample contains *E. amylovora* bacteria.

If the 381 - 399bp fragment is absent, your sample does not contain *E. amylovora* bacteria.

The *E. amylovora* - fragment might be sequenced if further investigation of the DNA sequence is necessary. The shorter 139bp fragment does not disturb this sequencing process. However, primers and nucleotides should be eliminated before sequencing with a simple cartridge purification. Microsynth will provide such a purification and sequencing service upon request.

Appendix 1: Recommended Taq Polymerases

FastStart Taq DNA Polymerase, Roche, Order number 12 158 264 001 (50 Units; different numbers for higher quantities)

HotStar Taq DNA Polymerase, Qiagen, Order number 203203 (250 Units) or 203205 (1000 Units)

AmpliTaq Gold, Applied Biosystems, Order number N808-0240 or N808-0241

The following Taq Polymerase enzymes have been tested at Microsynth and do not work with this buffer system:

Taq DNA Polymerase, Roche, Order number 11 146 165 001 (100 Units; different numbers for higher quantities)

Taq DNA Polymerase, Qiagen, Order number 201203

Taq DNA Polymerase, Invitrogen, Order number 18038

Taq DNA Polymerase recombinant, Invitrogen, Order number 10342

Taq DNA Polymerase, Sigma, Order number D1806 and D8187

Taq DNA Polymerase, Promega, M1661

Taq DNA Polymerase, Amersham 27-0799

Appendix 2: This PCR assay has been successfully tested with samples from the following countries:

Canada, Cyprus, Czech, Egypt, France, Greece, Ireland, New Zealand, Slovakia, Spain, Sweden, Switzerland, The Netherlands, Turkey, United Kingdom, United States

Appendix 3: Fire blight hosts tested:

Crataegus, Cotoneaster, Eryobotria, Malus, Pyracantha, Pyrus

### **Appendix for high throughput: Special rack and 1ml – water tubes**

For high throughput analysis, Microsynth will deliver tubes prefilled with water and special racks. This rack facilitates the dilution step of the heated extract.

Per rack, put up to 24 heated extracts and water tubes for dilution in a 3 x 8 array.

Centrifuge the rack shortly (5 sec. at low speed 1000rpm) to eliminate the liquid in the lid.

Make the dilution using a 8-channel pipettor.

Mix the dilution carefully (for a good mixture in full tubes, the air bubble should move from top to bottom)

Centrifuge the rack shortly to eliminate the liquid in the lid.

Transfer 1 µl of the diluted extract into the PCR tubes (with single pipettor or 8-channel pipettor).

Material for this step:

Expandible 8-channel pipettor (Matrix)

Centrifugation rotor for microplates

### Customer's feedback

Your feedback is very important for us. Upon request, Microsynth will develop a kit which is based on Real-Time PCR.

Version 1.05