LAB TRAINING - EUPHRESCO PHYTFIRE
LISBON, 3-4 OCTOBER 2012

October 3.

09:00-10:00 Processing of samples of bacterial suspensions and of plant tissue spiked with bacterial suspensions (EPPO, 2004).

The samples will be prepared in Spain with a general procedure **(EPPO, 2004)** valid for downstream applications such as: isolation, serological tests and PCR analyses, and will be provided for the training.

Plant material: healthy pear shoots, leaves, fruits, flowers.

Bacterial suspensions. A suspension of 10^6 cfu/ml of *E. amylovora, E. pyrifoliae* and *E. piriflorinigrans* treated at 100 ºC for 10 min, will be used as positive controls for the PCR amplifications.

10:00-11:00 *E. amylovora, E. pyrifoliae* and *E. piriflorinigrans* detection and identification.

A) DNA extraction (Taylor *et al*., 2001) for *E. amylovora* and *E. pyrifoliae*.

This extraction step is required for the samples of processed plant material, but not for the samples of bacterial suspensions. Take a lot of care in avoiding contamination among the samples during the different steps of extracting DNA.

- Spin down the samples of processed plant material to collect them at the bottom of the tubes and prevent contamination.
- Mix 200 µl of each sample with 500 µl of extraction buffer (provided, see recipe below).
- Incubate tubes for 15 min at room temperature.
- Perform subsequent analysis with the quantity required according to the amplification protocol. Alternatively, store at -20 ℃ until use.

Extraction Buffer		
Components	**Final concentration**	**for 1000 ml**
NaCl	140 mM	8.19 g

KCl	50 mM	3.73 g
Tween 20	0.05 %	0.5 ml
PVP 10	2 %	20 g
BSA	0.4 %	4 g
Distilled water		Up to 1000 ml

B) DNA extraction for *E. piriflorinigrans* (DNeasy Plant mini Kit – QIAGEN)

- Mix 200 µl of each processed plant material with 400 µl Buffer AP1 and vortex vigorously.
- Incubate the mixture for 10 min at 65℃. Mix 2 or 3 times during incubation by inverting tube.
- Spin down each tube to collect the sample at the bottom and prevent contamination.
- Add 130 µl Buffer AP2 to the lysate, vortex and incubate for 5 min on ice.
- Spin down each tube to collect the sample at the bottom and prevent contamination.
- Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 20,000 x g (14,000 rpm).
- Transfer the flow-through fraction from the previous step into a new microcentrifuge tube without disturbing the pellet.
- Add 800 µl of Buffer AP3/E to the cleared lysate, and mix by pipetting. (Ensure that ethanol has been added to Buffer AP3/E as it is indicated on the bottle).
- Pipet 700 µl of the mixture from the previous step, into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 6000 x g (8000 rpm), and discard the flow-through. Reuse the collection tube in the next step.
- Repeat the previous step with the remaining of the sample.
- Place the DNeasy Mini spin column into a new 2 ml collection tube, add 500 µl Buffer AW, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard the flow-through and reuse the collection tube in the next step. (Ensure that ethanol is added to Buffer AW as it is indicated on the bottle).
- Add 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 20,000 x g (14,000 rpm) to dry the membrane. (Following the centrifugation, remove the DNeasy Mini spin column from the collection tube carefully so the column does not come into contact with the flow-through, as this will result in carryover of ethanol).

- Transfer the DNeasy Mini spin column to a 1.5 ml microcentrifuge tube and pipet 100 µl Buffer AE (pre-heated at 65 ^{o}C) directly onto the DNeasy membrane.
- Incubate for 5 min at room temperature (15–25℃), and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.
- Discard the column and store the DNA extraction at -20ºC.

Real time PCR for *E. amylovora* (Pirc *et al*., 2009) (*amsC gene*)

Primers and probe:

Ams116F (5'- TCCCACATACTGTGAATCATCCA -3')

Ams189R (5'- GGGTATTTGCGCTAATTTTATTCG -3')

Ams141T (5'-FAM-CCAGAATCTGGCCCGCGTATACCG-BHQ1-3')

Amplicon size: 74 bp.

Prepare thermal cycler

Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions. Set cycling parameters:

2 min at 50 ℃	UNG activation step*
10 min at 95 ℃	polymerase activation
40 cycles of 15 s at 95 ℃ 1 min at 60 ℃	 DNA denaturation annealing and extension

*depending on the mastermix. This step will not be used in the training.

Assign samples to locations on a plate. Assign detectors to your samples (FAM as a reporter dye, BHQ1 as quencher – in Applied Biosystems instruments such quenchers are marked as non-fluorescent).

Save file.

Prepare and aliquot reaction mixture

Thaw primers and probe mixture completely, vortex briefly and spin down.

Prepare reaction mixture:

Component	**Final concentration**	**10 µl reaction**	**20 µl reaction**	**25 µl reaction**
		1 reaction	**1 reaction**	**1 reaction**
Ultrapure water		1 µl	2 µl	2.5 µl
2xTaqMan Universal PCR Master Mix	1x	5 µl	10 µl	12.5 µl

Primer Ams 116F [10 pmol/µl]	0.9 µM	0.9 µl	1.8 µl	2.25 µl
Primer Ams189R [10 pmol/µl]	0.9 µM	0.9 µl	1.8 µl	2.25 µl
Probe Ams141T [10 pmol/µl]	0.2 µM	0.2 µl	0.4 µl	0.5 µl
Aliquot to		**8 µl**	**16 µl**	**20 µl**
Sample DNA		2 µl	4 µl	5 µl

Calculate the number of reactions to be performed. Samples should be analyzed in one reaction each. Take into account pipetting errors and prepare reaction mixture for one sample more than actually needed. In a clean eppendorf tube mix water, universal master, primers and probe.

Aliquot the reaction mixture in suitable reaction tubes or PCR plates and add sample DNA. Close reaction container (close tubes or cover plates with optical foil). Centrifuge to collect all reaction components at the bottom of reaction container and remove bubbles, by centrifuging 3 min at 1000 x g. Process immediately or store in dark at 2-8 ^{o}C.

Real time PCR for *E. amylovora* (Gottsberger *et al.*, 2010) (chromosomal hypothetical protein 'AMY1267' of the strain Ea273).

Primers and probe:

hpEaF: (5'-CCGTGGAGACCGATCTTTTA-3')

hpEaR: (5'-AAGTTTCTCCGCCCTACGAT-3')

hpEaP (5'-FAM-TCGTCGAATGCTGCCTCTCT-MGB-3')

Amplicon size: 138 bp

Prepare thermal cycler

Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions. Set cycling parameters:

2 min at 50 ℃	UNG activation step*
10 min at 95 ℃	polymerase activation
40 cycles of 15 s at 95 ℃ 1 min at 60 ℃	 DNA denaturation annealing and extension

*depending on the mastermix. This step will not be used in the training.

Assign samples to locations on a plate. Assign detectors to your samples (FAM as a reporter dye, MGB as quencher – in Applied Biosystems instruments such quenchers are marked as non-fluorescent).

Prepare and aliquot reaction mixture

Thaw primers and probe mixture completely, vortex briefly and spin down.

Prepare reaction mixture:

Component	Final concentration	20 µl reaction 1 reaction
Ultrapure water		6.9 µl
2xTaqMan PCR Master Mix	1 x	10 µl
Primer hpEaF [10 pmol/µl]	0,5 mM	1 µl
Primer hpEaR [10 pmol/µl]	0,5 mM	1 µl
Probe hpEaP [10 pmol/µl]	0,05 mM	0.1 µl
Aliquot to		**19** µl
Sample DNA		1 µl

Conventional PCR for *E. amylovora* (Taylor *et al*., 2001) (probably of chromosomal origin).

Primers:

G1-F: 5‘-CCTGCATAAATCACCGCTGACAGCTCAATG-3‘
G2-R: 5‘-GCTACCACTGATCGCTCGAATCAAATCGGC-3‘

Amplicon size: 187 bp

Mastermix

	1 reaction of 25 µl	
	for bacterial suspensions	**for plant material**
Component	**Volume**	**Volume**
Ultrapure water	14.3 µl	14.1 µl
Buffer 10X	2.5 µl	2.5 µl
$MgCl_2$ [50 mM]	0.75 µl [1,5 mM]	0.75 µl [1,5 mM]
dNTPs [10 mM]	0.25 µl [0,1mM]	0.25 µl [0,1mM]
G1-F [10 pmol/µl]	1 µl [10 pmol]	1 µl [10 pmol]
G2-R [10 pmol/µl]	1 µl [10 pmol]	1 µl [10 pmol]

Taq polymerase [5 units/µl]	0.2 µl [1 unit]	0.4 µl [2 units]
Aliquot to	**20 µl**	**20 µl**
Add sample	5 µl	5 µl

Cycling conditions

PCR-steps	**Cycling**	**Temperature (°C)**	**Time**
initial denaturation	1	95.℃	3.min
denaturation	**40**	94.℃	30.sec
primer-annealing		60.℃	30.sec
extension		72.℃	1.min
final extension	1	72.℃	5.min
cooling	1	15.℃	∞

11:00-11:20 Coffee break.

11:20-11:30 LAMP techniques. Description. Web link:

http://loopamp.eiken.co.jp/e/lamp/index.html

11:30-13:00 LAMP amplifications. Mix preparations

LAMP kindly provided by Todd Temple (OSU, Corvallis, USA), personal communication.

LAMP primers for detection of *Erwinia amylovora* (*amsL* gene)

Name	**5' to 3' primer sequence**
ALB Fip	CTGCCTGAGTACGCAGCTGATTGCACGTTTTACAGCTCGCT
ALB Bip	TCGTCGGTAAAGTGATGGGTGCCCAGCTTAAGGGGCTGAAG
ALB F	GCCCACATTCGAATTTGACC
ALB B	CGGTTAATCACCGGTGTCA

Primers Fip and Bip are used at 2.4 µM, primers F and B at 0.2 µM final concentrations.

Melting temperatures for primers are between 58 to 60℃.

Put in a 0,2 ml PCR tube the following reagents (final volume 50 µl):

LAMP Reaction Mix:

			1 reaction of 50 µl
Component	**Initial conc.**	**Final conc.**	**Volume (µl)**
10X Thermopol buffer	10X	1X	5
dNTPs	10 mM	1 mM	5
$MgSO_4$	100 mM	4 mM	2
BSA	10 mg/ml	0.4 mg/ml	2
ALB FIP	100 µM	2.4 µM	1.2
ALB BIP	100 µM	2.4 µM	1.2
ALB F	10 µM	0.2 µM	1
ALB B	10 µM	0.2 µM	1
Bst DNA *polymerase*	8 U/µl	16 U/reaction	2
Water			24.6
Sample DNA			5

These components make up the reaction mix for one 50 µl reaction volume. All the reagents have to be mixed on ice, and finally add the template DNA. Incubate at 65ºC for 60 min. Remove tubes and allow them to cool for 10 sec. Observe tubes for the presence of a white precipitate (cloudy tube or a solid white precipitate at the bottom of the tube, indicates a positive amplification). A clear solution in the tube is considered as a negative reaction.

LAMP from Moradi *et al*., (2012).

This protocol will not be tested; it is detailed only for information of alternative protocols.

LAMP primers for detection of *Erwinia amylovora* (*amsH* gene)

Name	5’ to 3’ primer sequence
AMSHFIP	CCACCAGCGGCATTAATGGCATTTTTAACTTCAGGTCAGCAAGCG
AMSHBIP	GCAGACTGGCGCAATGTGGTTTTTCAGAGCCTGTAGGGAAACAG
AMSHF3	ACGTAACTGGCGAAGTGAC
AMSHB3	TGATTTTGCGACGGGTCAC
AMSHLF	AGGATAGTCAGGGGGACGTTG
AMSHLB	GCTAACGCATGACGGACGC

Put in a 0,2 ml PCR tube the following reagents (final volume 25 µl):

			1 reaction of 25 µl
Components	**Initial conc.**	**Final conc.**	**Volume (µl)**
10X Thermopol buffer	10X	1X	2.5
dNTPs	10 mM	1.2 mM	3
$MgSO_4$	100 mM	2mM	0.5
Betaine	5 M	1 M	5
AMSHFIP	100 µM	1.6 µM	0.4
AMSHBIP	100 µM	1.6 µM	0.4
AMSFH3	10 µM	0.2 µM	0.5
AMSHB3	10 µM	0.2 µM	0.5
AMSHLF	10 µM	0.4 µM	1
AMSHLB	10 µM	0.4 µM	1
Bst DNA *polymerase*	8 U/µl	8 U/reaction	1
Sample DNA			2
Water			7.2

Primers Fip and Bip are used at 1.6 µM, primers F3 and B3 at 0.2 µM, loop primers LF and LB at 0.4 µM final concentrations. All the reagents have to be mixed on ice, and finally add the template DNA. Incubate at 63ºC for 60 min

Blue LAMP (Austria) (Karl Stich and Heidi Halbwirth)

13:00-14:00 Lunch

14:00-14:30 Visualization of amplifications

Real time PCRs: fluorescence observation

LAMP Visualization.

14:30-15:00 Lateral flow device for *E. amylovora* (Brion Duffy)

15:00-17:00. Analysis of results: real time PCRs and LAMP amplifications

Electrophoresis of PCR products

October 4.

09:00-12:30 Detection and identification of *E. pyrifoliae* and *E. piriflorinigrans*

E. pyrifoliae

Conventional PCR 1 (Wensing *et al.*, 2011) (*pstS–glmS* region)

Primers:

EPPSGL1646 5‘-CAGCGCATCATAAGTGTACC-3‘
EPPSGL2698c 5‘-TCTGGAATATCGGCTCCGTA-3‘

Amplicon size: 1052 bp.

Mastermix

	1 reaction of 50 µl	
	for bacterial suspensions	**for plant material**
Component	**Volume**	**Volume**
Ultrapure water	36.3 µl	36.1 µl
Buffer 10X	5 µl	5 µl
$MgCl_2$ [50 mM]	1.5 µl [1,5 mM]	1.5 µl [1,5 mM]
dNTPs [10 mM]	1 µl [0,2 mM]	1 µl [0,2 mM]
EPPSGL1646 [10 pmol/µl]	0.5 µl [5 pmol]	0.5 µl [5 pmol]
EPPSGL2698c [10 pmol/µl]	0.5 µl [5 pmol]	0.5 µl [5 pmol]
Taq polymerase [5 units/µl]	0.2 µl	0.4 µl
Aliquot to	**45 µl**	**45 µl**
Add sample	5 µl	5 µl

Cycling conditions

PCR-steps	**Cycling**	**Temperature (°C)**	**Time**
initial denaturation	1	95.℃	3.min
denaturation		94.℃	30.sec
primer-annealing		57.℃	30.sec

extension	**40**	72.℃	1.min
final extension	1	72.℃	5.min
cooling	1	15.℃	∞

Conventional PCR 2 (Kim et *al.*, 2001) (*cps* region)

Primers:

CPS1 5'-CGCGGAAGTGGTGAGAA-3‘

CPS2c 5'-GAACAGATGTGCCGAGTA-3‘

Amplicon size: 1200 bp.

Mastermix

	1 reaction of 50 µl	
	for bacterial suspensions	**for plant material**
Component	**Volume**	**Volume**
Ultrapure water	35.3 µl	35.1 µl
Buffer 10X	5 µl	5 µl
$MgCl_2$ [50 mM]	1.5 µl [1,5 mM]	1.5 µl [1,5 mM]
dNTPs [10mM]	1 µl [0,2mM]	1 µl [0,2mM]
CPS1 [10pmol/µl]	1 µl [10 pmol]	1 µl [10 pmol]
CPS2c [10pmol/µl]	1 µl [10 pmol]	1 µl [10 pmol]
Taq polymerase [5 units/µl]	0.2 µl	0.4 µl
Aliquot to	**45 µl**	**45 µl**
Add sample	5 µl	5 µl

Cycling conditions

PCR-steps	**Cycling**	**Temperature (^oC)**	**Time**
initial denaturation	1	95 ℃	3 min
denaturation	**40**	94 ℃	30 sec
primer-annealing		57 ℃	30 sec
extension		72 ℃	1 min
final extension	1	72 ℃	5 min
cooling	1	15 ℃	∞

Real time PCR *E. pyrifoliae* (based on Wensing *et al.*, 2011) (*pstS–glmS* region)

Primers and probe:

EPPSGS1089Q 5’-GGTTACCGCGTTCGTATGAT-3’

EPPSGS1228Qc 5’-TTGTTGTCGTGAGCGCATAG-3’

EPPSGSprobe FAM 5’-AGGCAAAATAGGACAGTTTGGTGA-3’ BHQ (designed for the training course)

Amplicon size: 138 bp

Prepare and aliquot reaction mixture

Thaw primers and probe mixture completely, vortex briefly and spin down.

Prepare reaction mixture:

Component	**Final concentration**	**25 µl reaction** **1 reaction**
Ultrapure water		7.25 µl
2xTaqMan PCR Master Mix	1x	12.5 µl
Primer EPPSGS1089Q [10 pmol/µl]	0.4 µM	1 µl
Primer EPPSGS1228Qc [10 pmol/µl]	0.4 µM	1 µl
Probe EPPSGSprobe [10 pmol/µl]	0.1 µM	0.25 µl
Aliquot to		**22 µl**
add DNA		3 µl

Prepare thermal cycler

Switch on the thermal cycler in advance as per producer’s instructions to warm it up and stabilize conditions. Set cycling parameters:

10 min at 95 ℃	polymerase activation
45 cycles of 15 s at 95 ℃ 1 min at 60 ℃	 DNA denaturation annealing and extension

E. piriflorinigrans

DNA extraction from plant material using DNeasy Plant mini kit (QIAGEN) previously described.

Conventional PCR (Barbé *et al.*, 2012) (pEPIR 37 plasmid)

Primers: The sequences of the primers will be available after publication. They are based on sequences from the pEPIR 37 plasmid (Barbé *et al.*, in press).

p37F

p37R

Amplicon size: 255 bp.

Mastermix

	1 reaction of 50 µl	
	for bacterial suspension	**for DNA extraction**
Components	**Volume**	**Volume**
Ultrapure water	34.4 µl	34.2 µl
Buffer 10X	5 µl	5 µl
$MgCl_2$ [50 mM]	3 µl [3 mM]	3 µl [3 mM]
dNTPs [10mM]	0.4 µl [0,08 mM]	0.4 µl [0,08 mM]
P37F [10pmol/µl]	1 µl [10 pmol]	1 µl [10 pmol]
P37R [10pmol/µl]	1 µl [10 pmol]	1 µl [10 pmol]
Taq polymerase [5units/µl]	0.2 µl	0.4 µl
Aliquot to	**45 µl**	**45 µl**
Add sample	5 µl	5 µl

Cycling conditions

PCR-steps	**Cycling**	**Temperature (°C)**	**Time**
initial denaturation	1	94.℃	3 min
denaturation	**40**	94.℃	30 sec
primer-annealing		56.℃	30 sec
extension		72.℃	45 sec
final extension	1	72.℃	10 min
cooling	1	8.℃	∞

Real time PCR (Barbé *et al.*, 2012) (pEPIR 37 plasmid)

Primers and probe: The sequences of the primers and probe will be available after publication. They are based on sequences from the pEPIR 37 plasmid (Barbé *et al.*, in press).

p37trF

p37trR

p37

Amplicon size: 71 bp

Prepare and aliquot reaction mixture

Thaw primers and probe mixture completely, vortex briefly and spin down.

Prepare reaction mixture:

Component	12 µl reaction	25 µl reaction
	Bacterial suspension/DNA extraction	**Plant material (1/10 dilution in sterile distilled water) without DNA extraction***
Ultrapure water	1.2 µl	3.12 µl
2xTaqMan PCR Master Mix	6 µl	12.5 µl
Primer p37F [10 pmol/µl]	0.8 µl	1.95 µl
Primer p37R [10 pmol/µl]	0.8 µl	1.95 µl
Probe [10 pmol/µl]	0.2 µl	0.48 µl
Aliquot to	9 µl	20 µl
Sample	3 µl	5 µl

* DNA extraction is performed when 1/10 dilution of plant material samples, without DNA extraction, are negative. Then real time PCR is performed using the same reaction mixture as that designed for bacterial suspension.

Prepare thermal cycler

Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions. Set cycling parameters:

10 min at 95 ℃	polymerase activation
40 cycles of 15 s at 95 ℃ 1 min at 70 ℃	 DNA denaturation annealing and extension

Electrophoresis of PCR products

Agarose gels at 2 % (w/v)

Visualization and analysis of results:

- Electrophoresis of conventional PCR products in agarose gels at 2% (w/v)
- Comparison of amplification plots and CT values of real time PCRs
- visual score of LAMP amplifications

12:30-13:00 General discussion

References

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Barbé S, Llop P, Blom J, Cabrefiga J, Goesmann A, Duffy B, Smits T, Montesinos E, López MM. Complete sequence of *Erwinia piriflorinigrans* plasmids pEPIR 37 and pEPIR 5 and role of pEPIR 37 in pathogen virulence. *Plant Pathology* (in press).

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Pirc M, Ravnikar M, Tomlinson J, Dreo T (2009). Improved fireblight diagnostics using quantitative real-time PCR detection of *Erwinia amylovora* chromosomal DNA. *Plant Pathology* 58: 872–881.

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