The EPPO Standard PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* describes how validation should be conducted. It also includes definitions of performance criteria.

**Target Organism**

Erwinia amylovora

**Short description**

Real-time PCR detection of Erwinia amylovora using Ams amplicon

**Laboratory contact details**

National Institute of Biology, Department of Biotechnology and Systems Biology
Vecna pot 111, 1000 Ljubljana, Slovenia

**Date and reference of the validation report**

2011-10-20 - none

**Validation process according to EPPO Standard PM 7/98:**

Yes

**Reference of the test description**

PM 7/020(1)
http://www.euphresco.org/downloadFile.cfm?id=662

**Is the test the same as described in the EPPO DP?**

Yes

**Is the lab accredited for this test?**

No

**Plant species tested (if relevant)**

Different plant species that are host for Erwinia amylovora (Malus domestica, Pyrus communis, Cydonia oblonga, Cotoneaster sp., Chaenomeles sp., Crategus sp., Pyracantha sp., Sorbus sp.,)

**Matrices tested (if relevant)**

Symptomatic extracts were prepared from different plant parts with symptoms (subcortical tissues from twigs, leaves and fruits)

**List of methods used**

**Method for extraction / isolation / baiting of target organism from matrix**

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<tr>
<td>X</td>
<td>Extracts were prepared from symptomatic tissue; cut pieces were put into 1-2mL PBS buffer and incubated at room temperature for 10-20 minutes. Extracts were transferred to new eppendorf tubes. Three different extraction methods were performed and evaluated with real – time PCR 1- modified Llop et al. 1999 (Simple DNA) 2 – Dneasy Plant Mini Kit (Qiagen) 3 – QuickPick SML Plant DNA kit (Bionobile) The extraction method using QuickPick</td>
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SML Plant DNA kit (Bionobile) was further validated with real-time PCR with two additional experiments and calculated common LOD value

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<thead>
<tr>
<th>Method Category</th>
<th>Method Description</th>
<th>X Reason</th>
<th>LOD Value</th>
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<tbody>
<tr>
<td>Molecular methods, e.g. hybridization, PCR and real time PCR</td>
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<td>Real time PCR using Ams amplicon</td>
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<td>Serological methods: IF, ELISA, Direct Tissue Blot Immuno Assay</td>
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<td>Plating methods: selective isolation</td>
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<td>Bioassay methods: selective enrichment in host plants, baiting, plant test and grafting.</td>
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<td>Pathogenicity test</td>
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<td>Fingerprint methods: protein profiling, fatty acid profiling &amp; DNA profiling</td>
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<td>Morphological and morphometrical methods intended for identification</td>
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<td>Biochemical methods: e.g. enzyme electrophoresis, protein profiling</td>
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<td>Other</td>
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**Analytical sensitivity (= limit of detection)**

What is smallest amount of target that can be detected reliably? see full report

**Diagnostic sensitivity**

Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98

Specify the standard test Nested PCR (Llop et al., 2000)

**Analytical specificity**

Specificity value

Number of strains/populations of target organisms tested

423 strains of Erwinia amylovora were tested (list of tested strains are in Annex 1 and Annex 2)

Number of non-target organisms tested

97 non-target organisms or tissues (list of tested organisms and tissues are in Annex 3)

Cross reacts with (specify the species)

No cross reactivity was observed (for details see full report).

**Diagnostic Specificity**

Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test

Specify the standard test Not done
### Reproducibility

**Provide the calculated % of agreement for a given level of the pest (see PM 7/98)**

Reproducibility at: - LOD concentration: Three different symptomatic samples (previously tested as negative using standard methods) were spiked at LOD level (104 cells/ml) and aliquoted. Evaluation of reproducibility was tested in combination with QuickPick™ SML Plant DNA extraction method. Number of replicates were 7 (sample 1), 6 (sample 2) and 9 (sample 3). Sample 1: Average Cq value=33.81, SD=0.74, CV 2.21% Sample 2: Average Cq value=33.87, SD=0.82, CV 2.43% Sample 3: Average Cq value=33.68, SD=0.66, CV 1.97% 100% agreement for positive result. - Medium concentration: DNA was extracted from Erwinia amylovora suspension with medium level of concentration with QuickPick™ SML Plant DNA extraction method and aliquoted. Real-time PCR was performed 7 times. The average Cq values in all runs were 29.82 (SD 0.43 and CV 1.45%)- 100% agreement for positive results - High concentration: DNA was extracted from Erwinia amylovora suspension with high level of concentration with QuickPick™ SML Plant DNA extraction method and aliquoted. Real-time PCR was performed 7 times. The average Cq values in all runs were 23.05 (SD 0.44 and CV 1.92%)- 100% agreement for positive results.

### Repeatability

**Provide the calculated % of agreement for a given level of the pest (see PM 7/98)**

As we got very reproducible results in all tested concentrations (low/medium/high) in combination of different days and operators we didn’t test this separately.

### Test performance study

**Test performance study?**

Yes

**Include brief details of the test performance study and its output. If available, provide a link to published article/report**

2. Ring test 2010 (14 laboratories - Final report is not available yet)

In year 2009 real time PCR with Ams amplicon was first included in ring test among four project partners in Euphresco Erwindect project (final report of the project is available on webpage: [http://www.euphresco.org/downloadFile.cfm?id=662.](http://www.euphresco.org/downloadFile.cfm?id=662.))

To standardize the procedure of real time PCR as much as possible all chemicals were provided to all partners. For real time PCR different instruments were used among partners and different reaction volumes. Instruments and PCR condition used by different partners in real time PCR analysis are shown in table 4.3.2.6 in final report.

The ring test was composed of two parts:

Part 1: Testing of the DNA isolated by the partner 4 (QuickPick™ SML Plant DNA extraction method)

Results of the four laboratories were in high accordance (Table 4.2.3.7 in final report). No template control and DNA extracted from E. amylovora negative plant material gave negative results in all laboratories. The lowest concentration of target bacteria tested, corresponding to 1.8 x 10E3 cfu/mL...
plant extract was positive in all cases with minimum two out of three parallel reactions positive. Higher concentrations of E. amylovora were detected in all cases. This corresponds to specificity and sensitivity values of 1 with 0 rates of false positive and false negative results.

Part 2: Each partner (4) prepared their own non-spiked and spiked samples (final concentrations: 107, 106, 104, 103, 102 cfu/ml) using plant material sent out by Partner 2 and bacterial suspension of E. amylovora strain CFBP 1430. DNA from this material was isolated using modified Taylor DNA extraction.

Sensitivity in spiked pear sample was 10E3 cfu/ml, as expected from preliminary studies and theoretical limits of real time PCR method.

On spiked plant samples, prepared by ring-test partners, real time PCR had analytical sensitivity of 0.67 and specificity of 0.83.

False positive rate was 0.17, most likely due to contaminations during preparation of spiked samples especially as in one case also classical PCR was positive. It is generally accepted that the rooms for preparation of samples, extraction of DNA and real time PCR reaction preparation should be separated this could not be guaranteed by all ring-test partners. Based on experience of using real time PCR in routine analyses it is expected that by using proper procedure to minimize the risk of cross-contaminations the level of false positive results can be greatly reduced.

False negative rate - do we report it above LOD, this would be more informative was 0.33, due to samples with concentrations below theoretical and practical sensitivity of the method.

While symptomatic fire-blight samples with such low concentrations are not expected this the sensitivity can be further increased by analyzing a bigger volume of sample DNA.

In year 2010 real time PCR with Ams amplicon was included in ring test among 14 laboratories (AGES, Austria; CDR, Spain; IVIA, Spain; LRBV, Spain; LNPV, France; FS, Marocco; INRA, Marooco; NAK, The Netherlands; PD, The Netherland; MAF, New Zealand; RCPQ, Russia, NIB, Slovenia; UBFA, Serbia; OSU, USA). - Final report is not available yet .

**Other information**

**Any other information considered useful e.g. robustness, ease of performing the test, etc.**

Real-time PCR were in ring test 2009 performed on different PCR cycling instruments and reaction volume. Also different software for signal acquisition and analysis were used. Despite differences Ct values, efficiency of amplification and theoretical cycle threshold value for one copy detection were comparable in most cases (see in ERWINDECT final report Fig 4.3.2.1).

Real-time PCR (Ams assay) were transferred also to SmartCycler_instrument. Comparison of analytical sensitivity
was performed. All results are available in publication Tanja Dreö, Manca Pirc and Maja Ravnikar; Real-time PCR, a method fit for detection and quantification of Erwinia amylovora; http://www.springerlink.com/content/x27m424n1r9m73v8/

| The following complementary files are available online: | • [Detailed information on the validation of the Real-time PCR detection of Erwinia amylovora using Ams amplicon](http://www.springerlink.com/content/x27m424n1r9m73v8/) |