Project no: **502348**

Project acronym: **PORT CHECK**

Project title: **Development of generic ‘on site’ molecular diagnostics for EU quarantine pests and pathogens**

Instrument: **Specific targeted research or innovation project**

Thematic Priority: **8.1.B.1** ***Scientific Support to Policies - Sustainable management of Europe's natural resources***

Final Activity Report

Period covered: **March 2004 to August 2007** Date of preparation: **Nov 2007**

Start date of project: **March 2004** Duration: **3 years**

Project coordinator name: **Neil Boonham**
Project coordinator organisation name: **Central Science Laboratory**

1. Project Execution

Summary

The PORT CHECK project was a combined RTD and demonstration activity between a number of RTD laboratories, SME laboratories and end user laboratories (NPPO) with the aim of developing and testing a portable real-time PCR testing platform for some key EU quarantine organisms. PORT CHECK aimed to deliver the tools and procedures to allow EU member state Plant Health competent laboratories and inspection services to perform molecular diagnostic assays "on-site" and at points of entry. The project developed and evaluated real-time PCR (TaqMan and CyCleave) assays for a number of key harmful organisms, including the sudden oak death pathogen (*Phytophthora ramorum*) and the pinewood nematode (*Bursaphelenchus xylophilus*); and transferred these assays to field portable real-time PCR platforms.

The PORT CHECK project consortium performed R&D to develop every part of the process involved in on-site detection. From sampling and sample homogenisation, through simplified DNA extraction, set up of real-time PCR detection reactions using field-stabilised reagents to actual testing and interpretation of the results. The protocols developed were validated using infected material and were compiled into a comprehensive publicly available booklet for the detection of 12 different quarantine pathogens and pests. By working with SME companies prototype equipment and kits were made available to the consortium (and post project to the wider community) to enable homogenisation of samples, (partner 7, Bioreba), DNA extraction (partner 6, DRI/Invitrogen), field stable reagents (partner 42, TaKaRa BioEurope) and portable real-time PCR (partner 8, Cepheid Europe).

PORT CHECK then put the technology in the hands of thirty-two NPPO laboratories and inspection services for evaluation. The results from the method evaluation study showed that non-specialist users could generate results equivalent to those achieved in the laboratory in less than two hours, and that this could be done remotely from the laboratory.

Sample homogenisation

Following evaluation of all of the available sample homogenising equipment available, a prototype 'in field' homogenisation device was built (see figure below). The device uses a sealed grinding head to homogenise samples (0.5g of material in 5ml of DNA extraction buffer) within a sealed disposable sample bag. The aim of this design is to enable samples to be taken within the bag that the sample is then homogenised in; the use of disposable bags will prevent sample-to-sample contamination and also negate the need to decontaminate the homogenisation device between samples. The device can be powered using mains electricity, using a car battery or using its own internal battery pack. The sample-grinding period is variable and can be set in advance to an appropriate period for the material in question (leaf, wood etc.). The following pictures illustrate the prototype device.

A **B** **C**

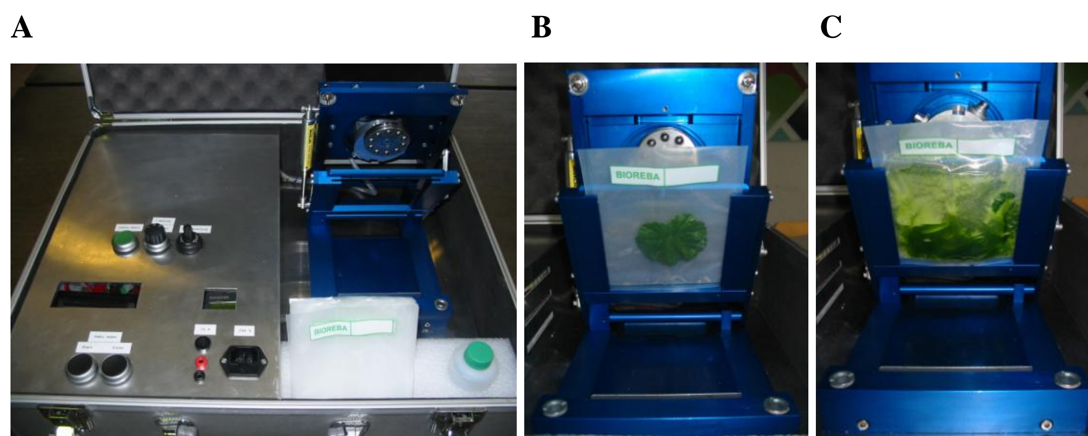


Figure: Photographs of the sample homogenisation prototype illustrating (A) the device with controls on the left and controllable grinding apparatus on the right, (B) a sample before homogenisation and (C) a samples after homogenisation.

Nucleic acid extraction

For the initial DNA extraction work rhododendron leaf material was chosen as the model system for developing protocols, since it is the most commonly encountered host of *Phytophthora ramorum* in the EU. In addition to being an important host Rhododendron is a difficult plant matrix to extract DNA from and if a successful purification protocol was developed for Rhododendron, it is likely that it could be used and (with further validated) on other plant types. A plant purification protocol was developed for Rhododendron based on the ChargeSwitch® Magnetic Bead technology. The extraction of DNA was monitored using the detection of a plant gene

(Cytochrome oxidase I) using a real-time PCR assay (based on TaqMan chemistry) developed previously, the quality of the extracted DNA was also monitored using gel electrophoresis. The results showed that Rhododendron DNA could be purified from fresh rhododendron leaves without any degradation observed and could be successfully amplified using real-time PCR (see figure below).

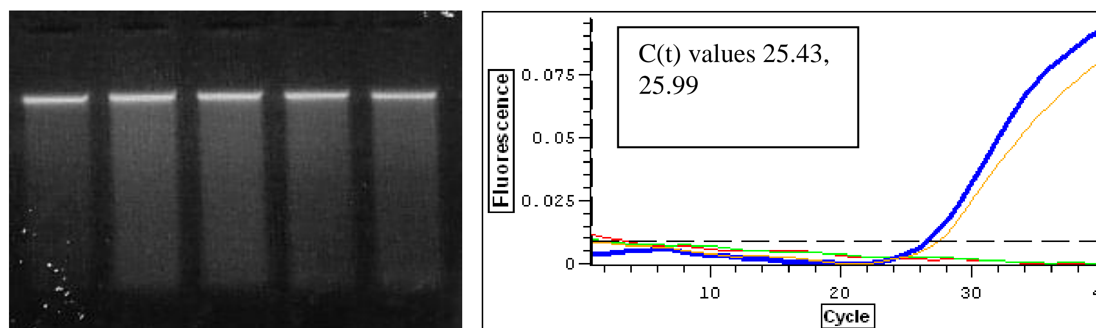


Figure: Gel picture (left) showing six replicates of Rhododendron leaf DNA purified using the DNA extraction kit based on Chargeswitch magnetic beads. Graph (right) showing the successful amplification (using real-time PCR) of DNA purified using the ChargeSwitch kit.

The developed purification protocol was further tested on other plant types. The results showed that the protocol could be used successfully for purification of DNA from other plant species. In addition the kit was used successfully for extraction of DNA from *Phytophthora ramorum* infected plant material (figure below).

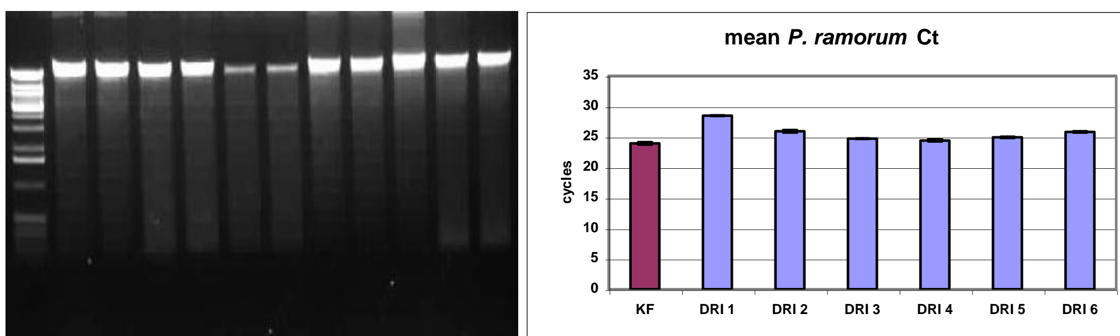


Figure: Gel picture (left) showing DNA extracted from cotton (lane 2-3), sunflower (lane 4-7), canola (lane 8-9) and corn (lane 10-12) using the ChargeSwitch extraction kit. Histogram (right) showing the Ct values following amplification (using real-time PCR) of *Phytophthora ramorum* DNA purified using the DNA extraction kit based on ChargeSwitch magnetic beads (blue) compared to a standard laboratory technique (red).

The method was validated on a range of sample types (see figure below) and gives equivalent amounts of high quality DNA for PCR amplification when compared with conventional laboratory based techniques. The new method does not require any laboratory equipment and extractions on eight samples can be performed in less than 20 minutes.

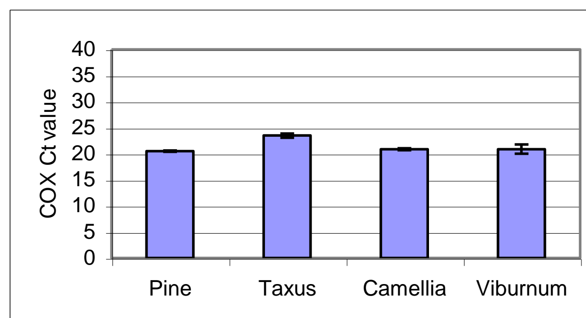
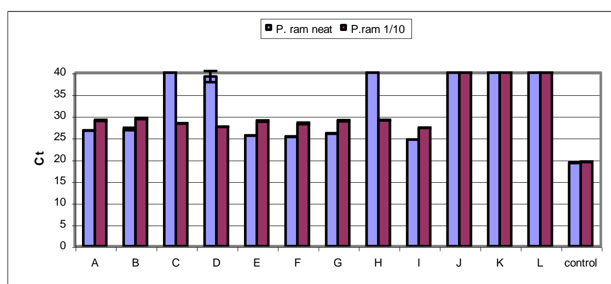


Figure: Histogram showing the Ct values for the detection of the internal control gene (Cytochrome oxidase) in DNA extracted from a range of plant species using the charge switch kit.

The method was shown to be effective for the extraction of all DNA from all the plant matrices of interest in the project. The figure below shows extraction of wood - the most challenging target in the project (François C., Castagnone C., Boonham N., Tomlinson J., Lawson R., Hockland S., Quill J., Viera P., Mota M. and Castagnone-Sereno P. (2007) Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, Bursaphelenchus xylophilus. Molecular Plant Pathology 8 (6): 803-809).

A **B**



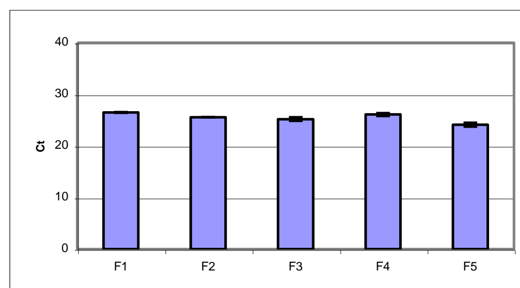


Figure: Figure showing real-time PCR data for extraction of DNA from wood samples. (A) Shows the extraction and detection of *Phytophthora ramorum* from beech samples (a-i) and oak samples (j-l). (B) Detection of Pine wood nematode DNA following extraction from 5 wood samples.

Real-Time PCR

Real time PCR assays were developed for all of the pathogens of interest in the project. The assays for the following pathogens were available either in the literature or within the consortium: *Phytophthora ramorum, Thrips palmi Ralstonia solanacearum, Clavibacter michiganensis subsp. sepedonicus* and the internal control COX I. In addition assays for the following pathogens have also now been developed: *Synchytrium endobioticum, Meloïdogyne chitwoodi, M. fallax, Guignardia citricarpa, Globodera pallida*, *G. rostochiensis* and *Bursaphelenchus xylophilus* (Walsh, K., Boonham, N., Barker I, Collins D. (2005) Development of a sequence specific real-time PCR to the melon thrips *Thrips palmi.* Journal of Applied Entomology 129 (5): 272-279). All assays were validated within the project and a database of the sequences for all the primers and probes, the location in the target sequences, the labels used in real-time PCR, concentration of primers and probes used, the type of internal control assay used and current extraction protocols has been compiled and is available via the project web page. The figure below shows an example of detection of one of these pests.

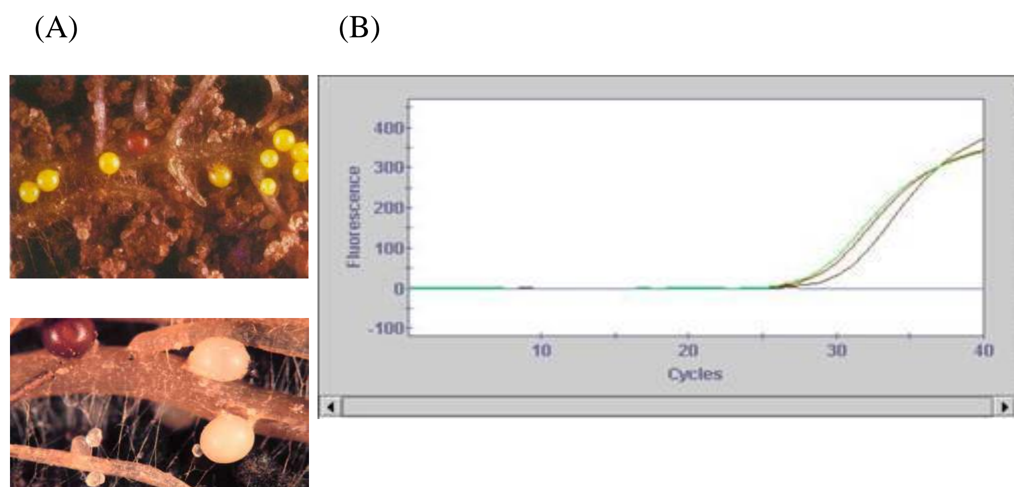


Figure: Illustrating (A) the cysts of *G. rostochiensis* and *G. pallida* and (B) the specific detection of Globodera using a real-time PCR based assay on TaqMan.

The two main pathogen targets within the project are *Phytophthora ramorum* and *Bursaphelenchus xylophilus.* Whilst assays already existed for *P ramorum* within the consortium for use with laboratory based equipment a completely new assay has been under development for the pine wood nematode (*B xylophilus).*

Phytopthora ramorum

For *Phytophthora ramorum* a number of assays were developed and validated within the PORT CHECK project. The conclusions of the work show that techniques based on Scorpion probes and Cycling Probe Technology (CPT) are considerably faster to generate a result than techniques based on TaqMan chemistry, reducing the length of an assay by more than half (Tomlinson J., Barker I., and Boonham N (2007) Faster, simpler, more specific methods for improved molecular detection of *Phytophthora ramorum* in the field *Applied and Environmental Microbiology* 73 (12): 4040-4047 & Tomlinson J. A., Boonham N., Hughes K. J. D., Griffin R. L., and Barker I. (2005) On-Site DNA Extraction and Real-Time PCR for Detection of Phytophthora ramorum in the Field. *Applied and Environmental Microbiology* 71: 6702-6710.). The assay based on CPT (Cycleave assay) was further tested for specificity and sensitivity against a range of species of Phytopthora and dilution series of DNA. The results show the assay to be completely specific and of an equivalent sensitivity to assays based on TaqMan chemistry. Following successful validation this assay was formulated into an ambient stable two part format (i) primer/probe beads and (ii) liquid reagent for use in the ringtesting.

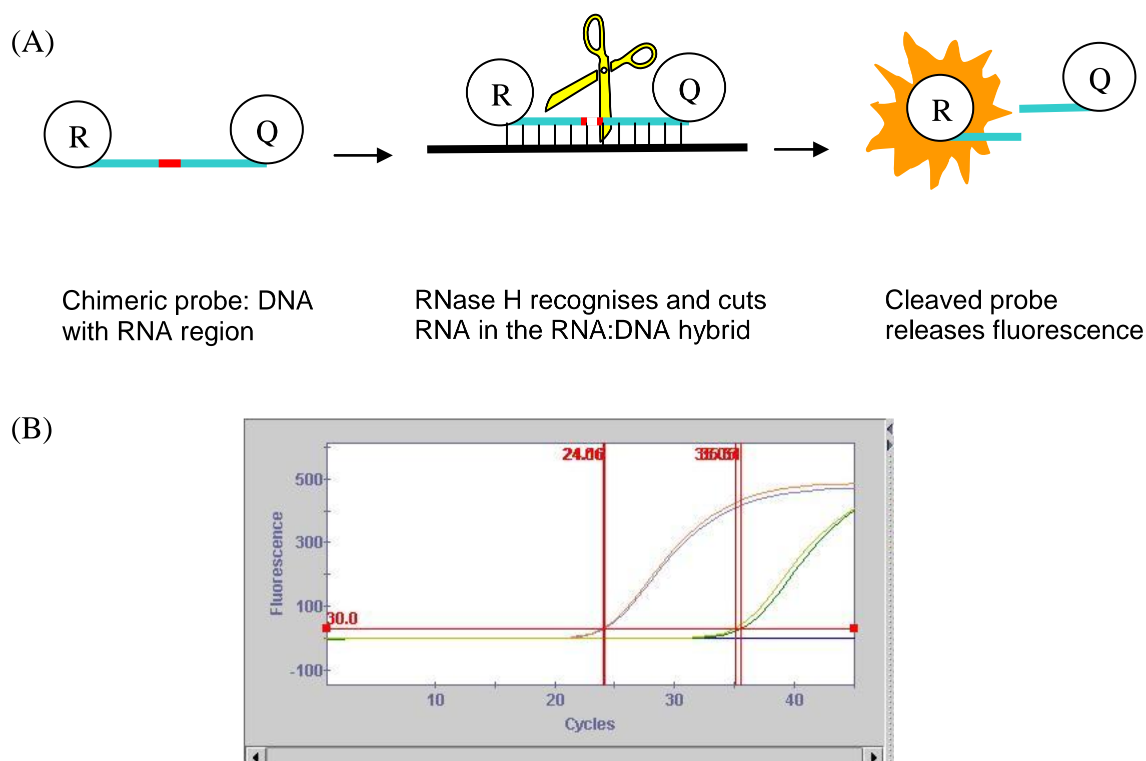


Figure: Illustrating (A) the mode of action of Cycling Probe Technology (CPT) and (B) the specific detection of *P ramorum* using CPT assay.

Bursaphelenchus xylophilus

For the pine wood nematode (*B xylophilus)* work concentrated on development of a real-time PCR assay targeted to the nematode satellite DNA. The assay was shown to give sensitive detection of *B xylophilus* both in single and mixed infestations. The assay was shown to be specific for *B xylophilus* and showed no cross reaction with the closely related *B mucronatus.*

The new PORT CHECK assay was tested on infested wood samples alongside existing 'conventional' methods based on the Baermann funnel isolation and enumeration. The results (table below) show that the new method gave results equivalent to the conventional technique and had a predicted limit of detection much lower than could be achieved using conventional techniques.

Table: Results from Baermann funnel isolation and enumeration, compared with Ct values generated following testing of DNA extracted using the CST method using real-time PCR.

Sample			Isolation	Ct
P3-T-36	Trunk 1.5-2 cm dia	'with lots of nematodes'	70 adults 68 juveniles	~18
P3-Bx-28	Trunk 1.5-2cm dia	'with nematodes but not so high number'	7 adults 3 juveniles	~24
P3-T-36	Small branches <0.5cm dia	'few nematodes'	10 adults 8 juveniles	~26*

Ringtesting

The protocols developed for all of the target pathogens were compiled into a comprehensive protocol booklet, including a 'quick reference card' for the CST extraction protocol. In addition a specific protocol for the detection of *Phytophthora ramorum* was drafted, detailing every stage of the process. The first ringtest (a method performance study) was also completed by all the partners in the project. This ringtest consisted of 16 freeze dried sap samples (14 positive and 2 negative) supplied to the testers blind; in total 21 testers completed the ringtest. The results (see below) show that the method has a diagnostic sensitivity of 98% and a diagnostic specificity of 90%, which is equivalent to, published laboratory methods (Hughes KJD, Tomlinson

JA, Griffin RL, Boonham N, Inman AJ, Lane CR (2006) Development of a one-step real-time polymerase chain reaction assay for diagnosis of *Phytophthora ramorum*. Phytopathology (9): 975-981).

Table: A two x two contingency table showing the results of the first port check ring test. The results show that the method has a Diagnostic sensitivity (A/A+C) = 98% and Diagnostic specificity (D/D+B) = 90%, when a positive/negative cut-off of cycle number 35 is introduced.

		True result		
		+	**-**	**Total**
PortCheck result	+	178 A	B 7	185
	-	C 2	D 65	67
	Total	180	72	252

An assay specific for *Phytophthora ramorum* based on CyCleave chemistry (TaKaRa) was developed; this reagent was formulated into a field stable reagent and used to complete the ringtesting. A further reagent based on liquid reagents has been developed for commercialisation by partner 42. This reagent was validated for sensitivity and specificity such that the PortCheck protocol could be included in the EPPO protocols (http://www.eppo.org/STANDARDS/standards.htm).

The second ringtest was completed by all the partners in the reporting period. The aim of this ringtest was to do an 'in-field' evaluation of the protocols developed using samples and assays of interest to the ringtester. In each case the tester was to complete the testing of the samples by the conventional methods used in the laboratory, this way the portcheck methods could be compared to a diverse and varied selection of methods.

Eleven of the thirteen available tests were used. None of the partners analysed samples with the *T. indica* or *G. citricarpa* assay. By combining all of the results together a comparison was made of the PortCheck protocols compared with conventional diagnostics used in each of the laboratories. The aim of the analysis was

to investigate the outcome of these protocols in the case where they were performed by an inspector prior to potentially sending samples to a laboratory, which may be performed in different ways depending on the scenario.

The results show that the PortCheck method has a positive predictive value (percentage of samples positive using the PortCheck method that later turn out to be positive in laboratory confirmation) of 89% and a negative predictive value (percentage of samples negative using the PortCheck method that later turn out to be negative in laboratory confirmation) of 86%.

Evaluation of these results is dependent on the scenario under which the inspector has performed the tests. In both scenarios below the aim is to empower the inspector to take more rapid action (on positive samples in the first and negative samples in the second), both have the additional benefit of reducing the sample burden in the laboratory; both have a cost of miss-diagnosis.

1. If the inspector has taken all the samples as suspicious (for example showing symptoms of disease) and used the PortCheck methods as a confirmatory test, sending to the laboratory only those samples that are negative (i.e. still suspicious). In total 39% of the samples will be ‘diagnosed’ in the field; of these 259 samples 11% would turn out to be false positive samples. The laboratory will as a result receive only 69% of the total suspicious samples to test. Assuming the laboratory test is infallible; the overall system will give a false positive result 4% of the time.

2. If the inspector were screening samples, sending to the laboratory only those samples that are positive for confirmation. In total 61% of the samples will be ‘diagnosed’ in the field, of these 422 samples 14% would turn out to be false negative samples. The laboratory will receive only 41% of the total samples to perform confirmatory testing. Assuming the laboratory test is infallible; the overall approach would give false negative results 9% of the time.

The first approach would be conservative and may be useful if the aim is to prevent the introduction of a damaging pathogen into a new area, accepting some false

positives as a risk. The latter may be more appropriate for post entry containment, accepting some false negatives as a risk.

Table: Contingency table showing the comparison of all samples tested using the PortCheck methodology compared with the results from the ‘currently used’ method of the laboratory performing the testing for a total of 11 pathogens/pests. The positive predictive values = A/(A +D) and negative predictive values = D/(C+D).

	Current methods			
PortCheck methods		+	-	Total
	+	230 *A*	*B* 29	259
	-	59 *C*	*D* 363	422
	Total	289	392	681

2. Dissemination and Use

Section 1 - Exploitable knowledge and its Use

Overview table

Exploitable Knowledge (description)	Exploitable product(s) or measure(s)	Sector(s) of application	Timetable for commercial use	Patents or other IPR protection	Owner & Other Partner(s) involved
Primer / probe sequences for: *Phytophthora ramorum, Thrips palmi, Ralstonia solanacearum, Clavibacter michiganensis subsp. sepedonicus*	Diagnostic tests for: *Phytophthora ramorum, Thrips palmi Ralstonia solanacearum, Clavibacter michiganensis subsp. sepedonicus*	1. Statutory plant health testing labs. 2. Commercial plant pathogen testing labs	2008	Patents are unlikely to be commercially viable, however 'knowhow' relating to standard operating procedures and validation will be exploited.	Partner 1
Primer / probe sequences for: *Synchytrium endobioticum, Meloïdogyne chitwoodi, M. fallax, Guignardia citricarpa, Globodera pallida*, *G. rostochiensis*	Diagnostic tests for: *Synchytrium endobioticum, Meloïdogyne chitwoodi, M. fallax, Guignardia citricarpa, Globodera pallida*, *G. rostochiensis*	1. Statutory plant health testing labs. 2. Commercial plant pathogen testing labs	2008	Patents are unlikely to be commercially viable, however 'knowhow' relating to standard operating procedures and validation will be exploited.	Partner 2
Primer and probe sequences for: *Bursaphelenchus xylophilus*	Diagnostic tests for: *Bursaphelenchus xylophilus*	1. Statutory plant health testing labs. 2. Commercial plant pathogen testing labs	2008	Patents are unlikely to be commercially viable, however 'knowhow' relating to standard operating procedures and validation will be exploited.	Partner 3
Plans for prototype homogenisation equipment	Sample grinding equipment	Any plant testing laboratories	2008	Design rights will be protected if deemed appropriate by partner 7.	Partner 7
Modifications to extraction protocols / new formats and new reagents	CST extraction kits that have a wider applicability	Any lab involved in plant biotech	2007	Modifications to protocol are unlikely to need additional protection, but will	Partner 6

Exploitable Knowledge (description)	Exploitable product(s) or measure(s)	Sector(s) of application	Timetable for commercia l use	Patents or other IPR protection	Owner & Other Partner(s) involved
				be exploited in new kit protocols.	

Primer and probe sequences

Exploitable knowledge has been produced by Partners 1, 2, 3 and 43 in this area, by development of a suite of real-time PCR assays for all of the pathogens of interest in the project as detailed in the overview table. These assays are likely to be of interest in the first instance to laboratories carrying out statutory Plant Health diagnostic testing, potentially in the longer term to laboratories carrying out commercial plant pathogen diagnostic testing. In the short term the project consortium contains a majority of the laboratories in Europe involved in statutory plant health testing, and will be exploited initially in the ringtesting of this project. Within the project it is intended that some of these assays will be produced in an ambient stable format (for ease of use) by Partner 43, if successful this SME would be a possible commercial outlet for the reagents developed. There is still research and development work and validation work to be completed before any further assessments on barriers to commercialisation; market considerations etc. can be made. In addition Partner 43 designed a real-time PCR assay for *P ramorum* based on Cycleave chemistry, this assay will be produced in an ambient stable bead form for the ringtesting, if successful this SME would be a possible commercial outlet for the reagent.

On-site sample homogenisation equipment

Exploitable knowledge has been produced by partner 7 in this area. Bioreba (partner 7) was the first company worldwide to produce and commercialise ELISA reagents for plant virus diagnostics in 1980, they produce and sell a full solution for plant testing including not only reagents but also equipment needed for plant pathogen diagnostics. As such they have a proven track record in commercialisation of equipment of this kind. It is likely that when fully developed the equipment will be available commercially alongside the other homogenisation equipment in the company catalogue.

Extraction methodology

Exploitable knowledge has been produced by partner 7 in the area of DNA extraction from plant material. Extraction kits based on ChargeSwitch technology for the extraction of DNA from plant material are already commercially available from Partner 6. The modifications that have been made to the protocol in the first reporting period allow more efficient extraction from plant material from which DNA extraction is more difficult. The modifications can be incorporated into the protocols available with the current kits as alternative protocols, thus this knowledge gained will be exploited immediately.

Section 2 – Dissemination of knowledge

Overview table

Planned/actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
Mar 2006	Meeting Portcheck R&D partners	Research	EU	12	2
Apr 2006	Meeting working	Research	NL	20	2

Planned/actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
	group potato cyst nematode				
June 2006	BioTech2006 Switzerland	Research	CH	50	2
Aug 2006	Key note conference presentation: The 8th Conference of the European Foundation for Plant Pathology and the BSPP Presidential Meeting (The Royal Veterinary and Agricultural University, Copenhagen) 13th – 17th of August 2006.	Research	EU	100	1
Aug 2006	PortCheck workshop: The 8th Conference of the European Foundation for Plant Pathology and the BSPP Presidential Meeting (The Royal Veterinary and Agricultural University, Copenhagen) 13th – 17th of August 2006. (see pictures below)	Research	EU	100	1
Aug 2006	PortCheck training workshop, Central Science Laboratory, York, UK (29-31st August)	Project consortium and associated policy departments	EU	45	1
Oct 2006	PortCheck 'on-site' training and test: Hull Docks, Hull, UK (see pictures below)	UK Plant Health and Seeds Inspectors	EU	6	1

Planned/actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
Oct 2006	PortCheck training workshop, Central Science Laboratory, York, UK (9-10th October)	Project partners	EU	5	1
Nov 2006	Meeting wart disease	Research	EU	30	2
Nov 2006	Meeting working group potato cyst nematode	Research	NL	20	2
Nov 2006	Key Note presentation: The 7th Australasian Plant Virology Workshop Rottnest Island 8th - 11th November, 2006	Research	Worldwide	50	1
Dec 2006	Departmental talk presented 7th December 2007) The National Institute of Biology, Slovenia.	Research	Slovenian	25	1
Jan 2007	Submitted publication *Phytophthora ramorum*	Phytopathology	All		4,2
Jan 2007	Submitted publication *Guignardia citricarpa*	Journal of Phytopathology	All		4,2
Jan 2007	PortCheck workshop: Plant Health and seeds inspectors' technical conference, 15-17th January 2007, Chester, UK. (see pictures below)	Inspectors	UK	100	1
Feb 2007	Meeting Portcheck R&D + ringtest partners	Research	EU	30	2
2007	R.A. Mumford, R.Weekes & N. Boonham (2007).	Research	Worldwide	75	1

Planned/actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
	Making diagnostics work: getting it right, everytime for the right price! Keynote presented at EAPR Virology Conference, Aviemore, UK.				
2007	R.A. Mumford (2007). Advances in molecular diagnostics - new solutions for old problems. Invited lecture presented at Institute of Botany, Vilnius, Lithuania.	Research	Lithuania	30	1

Workshop photos:

PortCheck workshop: The 8th Conference of the European Foundation for Plant Pathology and the BSPP Presidential Meeting (The Royal Veterinary and Agricultural University, Copenhagen) 13th – 17th of August 2006.

PortCheck 'on-site' training and field sample testing: Wednesday 11th October 2006. Hull Docks, Hull, UK

PortCheck workshop: Plant Health and seeds inspectors' technical conference, 15-17th January 2007, Chester, UK.

Section 3 - Publishable results

1. François C., Castagnone C., Boonham N., Tomlinson J., Lawson R., Hockland S., Quill J., Viera P., Mota M. and Castagnone-Sereno P. (2007) Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, *Bursaphelenchus xylophilus*. *Molecular Plant Pathology* 8 (6): 803-809
2. Tomlinson J., Barker I., and Boonham N (2007) Faster, simpler, more specific methods for improved molecular detection of *Phytophthora ramorum* in the field *Applied and Environmental Microbiology* 73 (12): 4040-4047
3. Hughes KJD, Tomlinson JA, Griffin RL, Boonham N, Inman AJ, Lane CR (2006) Development of a one-step real-time polymerase chain reaction assay for diagnosis of *Phytophthora ramorum*. Phytopathology (9): 975-981
4. Mumford R.A., Tomlinson J., Barker I. & Boonham N. (2006). Advances in molecular phytodiagnostics - new solutions for old problems. European Journal of Plant Pathology 116: 1-19.
5. Tomlinson J. A., Boonham N., Hughes K. J. D., Griffin R. L., and Barker I. (2005) On-Site DNA Extraction and Real-Time PCR for Detection of *Phytophthora ramorum* in the Field. Appl. Environ. Microbiol. 71: 6702-6710.
6. Walsh, K., Boonham, N., Barker I, Collins D. (2005) Development of a sequence specific real-time PCR to the melon thrips *Thrips palmi.* Journal of Applied Entomology 129 (5): 272-279