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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PH0412
2. Project title: Detection and identification of Phytophthora ramorum and Phytophthoa kernoviae using simple field test kits
3. Contractor organisation(s): Central Science Laboratory, Sand Hutton, York, YO41 1LZ
4. Total Defra project costs (agreed fixed price): £
5. Project: start date .... 03 April 2006
   end date .... 31 May 2008

6. It is Defra's intention to publish this form.
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(a) When preparing SID 5s contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.
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(b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

The Plant Health & Seeds Inspectorate (PHSI) of Defra along with Forestry Commission staff currently use a simple disposable field test kit known as a Lateral Flow Device (LFD), for detecting *Phytophthora* sp in the field. Samples that are positive for *Phytophthora* are then sent to the laboratory for confirmation at the species level. This kit uses a monoclonal antibody (or blend of antibodies) that gives specificity to the genus level. This project aim was to develop new monoclonal antibodies specific to the species *Phytophthora ramorum* and *kernoviae.* The aim was to transfer any monoclonals developed into the LFD format and then complete validation in the laboratory and field along with inspectors. Furthermore, since it was understood that the development of species-specific antibodies would be very challenging, the aim was to combine genus-level detection using an LFD with species-level detection using molecular techniques (e.g. Real-time PCR), by recovering and amplifying DNA from positive LFD's. It was hoped that this approach would increase the speed of analysis, decrease the cost and confirmation would be performed on the same sample analysed using an LFD, rather than a related, yet different sub-sample, which is the current standard. This refinement was aimed at further reducing the small number of discrepant results between field and laboratory analysis.

A selection of *Phytophthora* sp isolates and related and unrelated (but ubiquitous) fungal pathogens (e.g. *Pythium, Botrytis*) were selected and their identities confirmed using real-time PCR (TaqMan) and ITS sequencing. Artificially infected plant material and soluble-protein extracts (SPE) were produced from these cultures and used throughout the project for the

generation of monoclonal antibodies (Mabs). SPE of *P.ramorum* (2266) and *P.kernoviae* (2166) and also molecular weight enriched fractions of each isolate were used as immunogens. All mice immunised developed an immune response to the antigens used.

Following cloning and screening, four Mabs (D050, D056, D062 and D064) were identified to have specificity toward *P.ramorum* isolates. Extensive optimisation and cross reactivity testing later showed that these Mabs were limited in their detection range. It was concluded that these Mabs were specific to proteins or other antigens present in *P.ramorum* only at certain times during its growth and were later excluded from this project. An investigation into whether the antigens were specific to a particular life cycle stage (e.g. spores) proved inconclusive.

A large panel of antibodies were produced which were raised toward *P.kernoviae*. Extensive testing showed these antibodies showed similar immunogen specificity and did not detect any other *Phytopthora* sp isolates, thus no *P.kernoviae* specific antibodies were produced.

Three genus-specific *Phytophthora* Mabs (D036, D037 and D051) were successfully produced. The specificity of these Mabs toward *Phytophthora* sp matched that the profile of the commercially available reagents (Neogen) although following ELISA studies, cross-reactions with *Pythium* sp were less marked.

In order to effectively select Mabs for LFD development, further characterisation was performed by sequencing the variable light chains of the antibody molecules as well as performing epitope-mapping studies. The aim was to discriminate significantly different Mabs, which had the same species specificity. Antigen characterisation studies using *P.ramorum* and *P.kernoviae* revealed that all three genus specific *Phytophthora* Mabs bound to different protein epitopes, which likely formed part of a larger glycoprotein molecule. This sequencing work highlighted that each Mab had different antibody light chains. Mabs D036 and D037 were most similar (99.6% identity) whilst D051 differed significantly with only 95% sequence identity. The sequence of the variable light chains had a 99.6% identity between Mab D036 and D037 i.e. 1 base pair difference; where as 95% identity was recorded between Mab D051 and D036 and D037 i.e. 13 base pair differences.

Mab D036 and D037 were chosen as the preferred antibodies for genus-specific *Phytophthora* detection following testing in ELISA. A validation was conducted using an optimised ELISA assay testing 5 *Phytophthora* sp isolates and 8 unrelated fungal isolates all on infected leaf material. All isolates were correctly identified and the ELISA assay was shown to be specific, sensitive and robust.

The genus-specific *Phytophthora* Mabs (D036, D037 and D051) were tested in various combinations i.e. membrane or latex bound, within the LFD format. The combination of Mab D036 bound to the membrane and Mab D037 bound to the latex gave the best results (consistent line intensity and low background within 5 minutes of the sample extract being

applied to the device) and this combination was used to produce prototype kits. Cross-reactivity testing of the prototype was tested in parallel with the existing LFD kit in terms of sensitivity and specificity. The same extraction method was used to extract leaf material sent to CSL by the PHSI for confirmatory *Phytophthora* sp testing. A total of 107 leaf samples (healthy and infected) were tested in comparing the two devices the prototypes gave 96% diagnostic sensitivity and 100% diagnostic specificity.

An integrated LFD/Molecular approach to enable genus-specific detection of *Phytophthora* positive field samples by LFD to be further assessed to *Phytophthora* species-specific detection within the laboratory was explored. DNA from field-positive LFD devices was successfully extracted from genus-specific *Phytophthora* positive LFD devices and incorporated into real-time PCR (TaqMan) techniques. This method accurately predicted the *Phytophthora* species with a predictive value of 92.6% when compared against conventional diagnostic laboratory methods; and a negative predictive value of 92.1% was achieved. This would allow for positive LFD's to be returned to the laboratory rather than returning plant material. The use of this technique could have several advantages, firstly exactly the same sample is tested using both the LFD and the molecular test, rather than achieving an LFD result, then re-sampling the material for a laboratory test. Secondly, the DNA of the device is stable, thus if held up in transit samples will not degrade in the way leaf material does. Finally, since the DNA extraction is completed on the LFD, the laboratory testing would be faster and less expensive in terms of both consumables and time.

Report drafted by: Kate Perkins, Gaynor Johnson, Sioban Ostoja-Starzewska and Neil Boonham

Additional work is presented in the Annexes

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

O1 M 1: **A panel of Phytophthora cultures will be produced on both plates and host plants.**

A panel of *Phytophthora* species were inoculated onto healthy rhododendron leaf material and synthetic media. Extracts from these cultures were used for immunisation, antibody screening assays and later for validation of Lateral Flow Device (LFD) and enzyme linked immunosorbent assay (ELISA).

O1 M2: **Identify and generate a panel of unrelated pathogens, which infect similar hosts within the UK for screening purposes**

A panel of pathogens was identified and cultured on plates and artificially inoculated onto leaf material. Species initially used for antibody screening assays were: *Phytophthora cactorum, P. citricola* (two of the most commonly encountered species), *P. lateralis* (morphologically and molecularly very similar to *P. ramorum*), *P. kernoviae, Pythium ultimum, Pythium intermedium* (related species, known to cross-react with existing *Phytophthora* antibodies) and *Botrytis cinerea* (common coloniser of plant tissue and representative of a 'true fungus'). In order to characterise any selected antibodies further, a broader panel of *Phytophthora sp*. isolates and other commonly associated plant pathogens: *Fusraium sp, Alternaria* sp and *Trichoderma* sp were cultured on artificial media.

O1 M3: Confirm identity of Phytophthora ramorum and Phytophthora kernoviae by RT-PCR

The identity of the *P. ramorum* and *P. kernoviae* isolates used in the study was confirmed using species-specific real-time PCR (TaqMan) assays. These isolates were then grown up in bulk in potato dextrose (PD) broth to produce antigens for immunisation. Cultures were set up in PD broth alone and PD broth amended with pieces of healthy rhododendron leaf or homogenised healthy rhododendron leaf.

O1 M4: Separate specific proteins by various methods, including MW filters and specific gels

P. ramorum and *P. kernoviae* was harvested from the broth cultures. The mycelium was rinsed in deionised water, snap frozen and ground under liquid nitrogen. Protein extracts were suspended in Phosphate Buffered Saline (PBS) and centrifuged to remove debris. Soluble proteins were separated using SDS poly-acrylamide gel electrophoresis (PAGE), and visualised using Coomassie Brilliant Blue.

In initial comparisons, no clear differences between either the number or size of the proteins present in the PDA or amended PDA were evident. However, the cultures in un-amended broth produced the highest concentration of protein and were characterised further.

Following western blot analysis, using *Phytophthora* genus specific antibodies proteins in the range 35-60kDa were identified. In order to enrich the crude extracts for these putative immuno-dominant proteins, molecular weight spin filters were used to separate the proteins into appropriate fractions. Filters with a MW cut off of 100kDa, 50kDa and 30kDa were used, resulting in fractions of >100kDa, 50-100kDa, 30-50kDa and <30kDa. These were separated on an SDS PAGE gel, western blotted onto nitrocellulose membranes, and probed with a cocktail of the genus specific anti-*Phytophthora* antibodies.

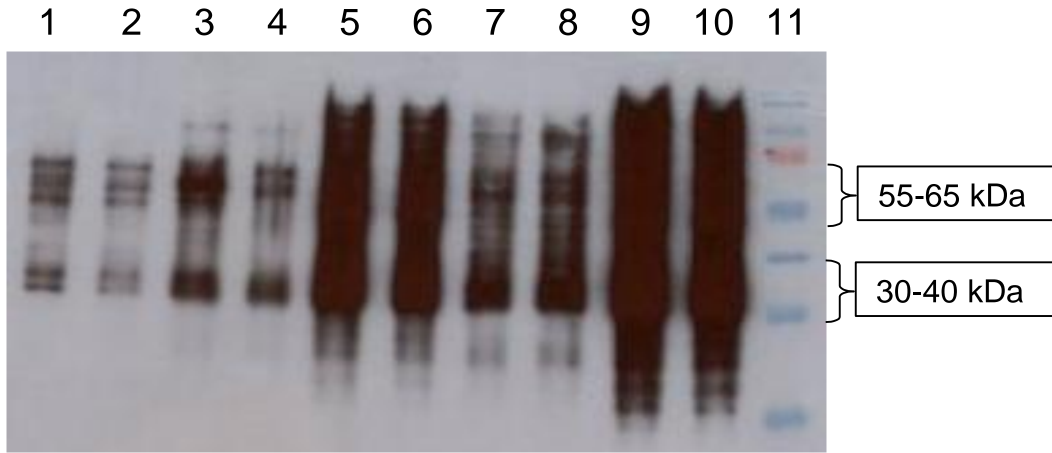


Figure 1a: A photograph of a western blot probed with a cocktail of anti-*Phytophthora sp*. monoclonal antibodies resolved using a chemiluminescent substrate.
Lanes 1 and 2: Whole *P. ramorum* – unfractionated
Lane 11: MW markers

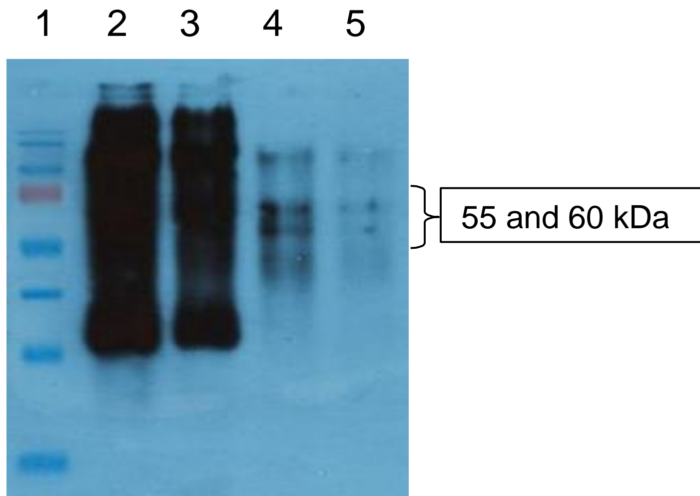


Figure 1b: A photograph of a western blot probed with a cocktail of anti-*Phytophthora sp*. monoclonal antibodies resolved using a chemiluminescent substrate.
Lane 1: MW markers
Lane 4: Fraction of 50-100kDa *P. ramorum*

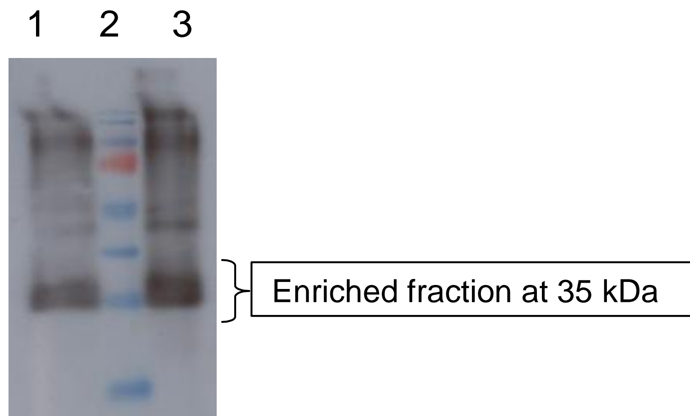


Figure 1c: A photograph of a western blot probed with a cocktail of anti-*Phytophthora sp*. monoclonal antibodies resolved using a chemiluminescent substrate.
Lane 1: Fraction of 30-50kDa
Lane 2: MW markers
Lane 3: Fraction of 30-50kDa

The results (figure 1) show that the protein extract can be fractionated using molecular weight cut off columns into two pools of proteins that are enriched for the proteins recognised by the genus specific Mabs (55-60 kDa and 35 kDa). These three immunogens were then used for panning the phage library and for immunisations (milestone 5 and 6). The first immunogen was a complete extract of soluble protein from the pathogen from which novel antibodies (potentially species specific) may be screened. The second and third are enriched pools of proteins containing antigens that are recognised by the genus specific monoclonal antibodies, It was hypothesised that these may be immunodominant proteins and may yield a larger panel of Mab's from which to select species specific reagents.

O1 M5: Proteins will be used to generate recombinant antibodies by biopanning phage libraries

Two recombinant antibody libraries were kindly supplied by the MRC Gene Service (Cambridge). The Tomlinson I library contains 147 x 10^8 variants, whilst the Tomlinson J library contains 1.37 x 10^8 (A Nissim, H R Hoogenboom, I M Tomlinson, G Flynn, C Midgley, D Lane, and G Winter (1994) Antibody fragments from a 'single pot' phage display library as immunochemical reagents.EMBO J. February 1; 13(3): 692–698). Both the I and J libraries were rescued and used in a range of selection experiments using *P ramorum* infected material and mycelia extracts as trapping antigens were performed.

The protocols from the MRC were followed; briefly these involve round of bio panning as described below. (a) The initial library of phage was rescued by infection with helper phage, which enables the packaging of mature phage particles expressing recombinant antibody fragments. (b) These phage particles were used for bio-panning, in which the particles were incubated in tubes coated with the antigen of choice; following washing to remove the unbound phage, the bound phage were eluted by altering the pH. (c) The recovered phage particles were infected onto *E coli* cells to produce a first round library. This library was then rescued and subsequent rounds of bio-panning completed.

A total of 16 different bio-panning selections were completed, each was repeated with both the I and the J libraries or subsets of these libraries in sequential selections. These involved positive panning against antigen from cultured pathogen or infected plant material, the aim being to select phage that bind to these antigens. In addition negative panning (against healthy plant material) was performed to remove phage expressing non-specific antibody fragments. These were performed in sequence, with up to three rounds of selection in each completed sequence. In each sequence only one of the three rounds would be negative selection, with the other two rounds being positive selection, or positive selection alone.

In one of these experiments polyclonal phage were tested using ELISA following three rounds of selection using the I library, performed as follows:

1. Against antigen from *P ramorum* infected leaf material (I-PR-33)
2. Negative selection against non-related fungal pathogens (I-PR-38)
3. Positive selection against antigen from *P ramorum* cultured material (I-PR-42)

The results show that the I library contain a large number of phage that bind to antigen within the plant material, whilst using fungal mycelial antigen (round1) gave and increase in phage binding to 'fungal' antigens including to the unrelated Botrytis negative control. Negative selection seemed to deplete the content of the library significantly (round 2) whilst taking this depleted library to a third positive selection round (round 3) with cultured material gave an increase in phage binding to the *P ramorum* infected leaf and also antigen from *P ramorum* culture.

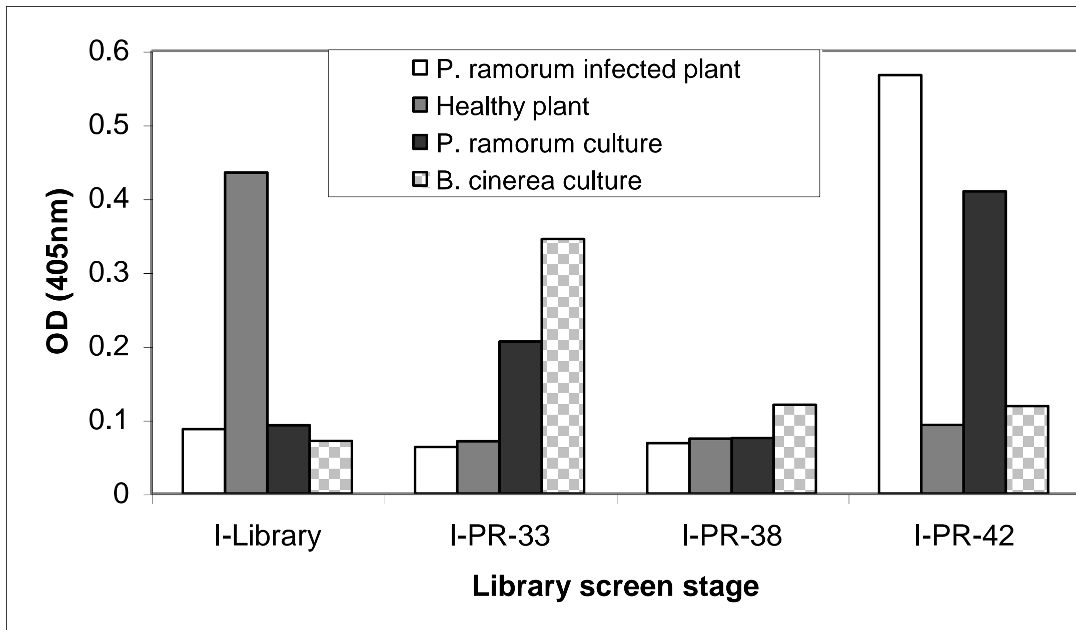


Figure 2: Histogram showing OD 405nm readings following PTA ELISA using polyclonal phage from various selection approaches.

Further work to select individual phage (monoclonal phage) from these libraries did not result in specific reagents being selected, the reasons for which are not known. It is possible that either not enough individual phage were screened in the selection process, or if there is complementary binding occurring in the polyclonal mixture meaning that individual phage are not able to bind.

O1 M6: Commence antibody production using the most suitable proteins

Mice were immunised with whole preparations and molecular weight fractions of *P. ramorum* isolate 2266 (50-100 kDa and a 30-50 kDa) and *P.kernoviae* isolate 2166 (50-100 kDa). CSL Standard Operating Procedures (SOP) for immunisation was followed, whereby each animal received 4 x 15ug doses of its respective immunogen at two-week intervals. The immune status of the animals was assessed at 8 weeks using polyclonal antiserum separated from blood taken from the tail of the animals. This polyclonal antiserum was assessed using Plate Trapped Antibody (PTA) ELISA and western blot analysis. Specificity profiles toward other fungal isolates were also evaluated. All immunised mice demonstrated good immune responses to the immunogen used; detection of the respective immunogen and proteins isolated from other *Phytophthora sp.* were recorded whilst no cross-reaction with unrelated fungal isolates was noted (see figures 2 and 3). Following a final immunisation (30ug of immunogen four days prior to fusion), extracted spleen cells from each *P.ramorum/ P.kernoviae* selected mouse were fused with immortal myeloma cell lines (SP2/0-Ag14 or P3/NS1/1-Ag4.1).

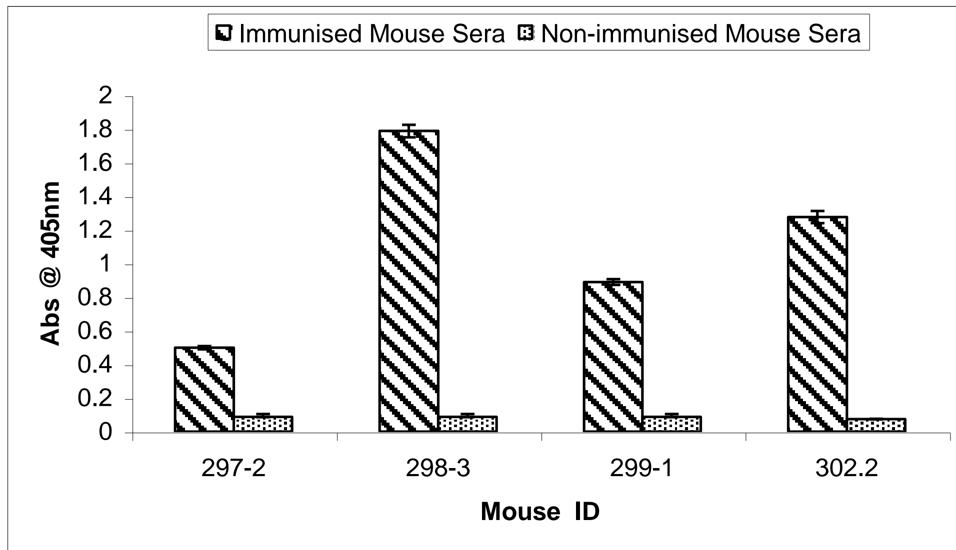


Figure 3: A histogram of absorbance values following a PTA-ELISA illustrating the immune response of mice immunised with *P.ramorum*.
Note: Dilution rate 1:4000 Stdev from 3 replicates

Table 1: A table showing the fusion number, mouse number and the immunogen used.

Fusion No	Immunogen Form	Mouse No
407	Whole P. ramorum	297.2
410	Whole P. ramorum	302.2
405	50-100 kDa	298.3
406	30-50 kDa	299.1

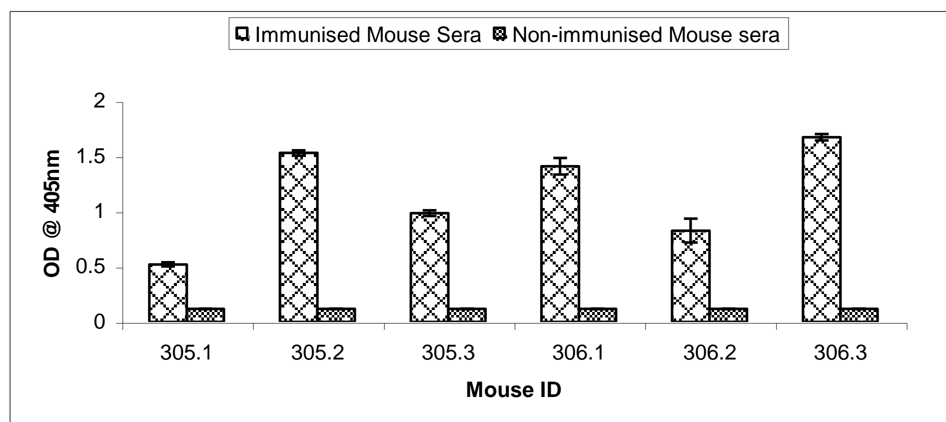


Figure 4: A histogram of absorbance values following a PTA-ELISA illustrating the immune response of all mice immunised with *P.kernoviae*
Note: Dilution rate 1:4000 Stdev from 3 replicates.

O2 M1: Screen all hybridomas with *P. ramorum* and *P. kernoviae* plus others selected in O1

The hybridomas were screened using PTA ELISA against soluble protein extracts of *Phytophthora ramorum*, *Phytophthora kernoviae* and other *Phytophthora sp.* plus many other non-related fungal isolates i.e. *Pythium* and *Botrytis*. Only those hybrids showing specificity toward *P. ramorum / P.kernoviae* and *Phytophthora* genus with no cross reaction toward the other non-related fungal isolates were taken forward for characterisation. Selected

hybrids were expanded into larger culture volumes and cloned using the limiting dilution technique. Extensive testing against a broader panel of isolates (approx 34) and against naturally and artificially infected plant material (e.g. Rhododendron) was performed.

Table 2 : Table showing the number of cell lines generated throughout the screening process.

	Fusion Number			
Screening	**405**	**406**	**407**	**410**
Number of Fusion Retests	166	62	44	16
Number of 1st Clones	28	9	4	4
Number of 1st Clone Retests	107	22	13	4
Number of 2nd Clones	16	10	4	0
Number of 2nd Clone Retests	64	40	16	0
Development				
Number selected for purification	16	10	4	0
Identification number	D035-D050	D051-D060	D061-D064	-
No: selected for characterisation	3	2	2	-

A *Phytophthora* genus Mab (Neogen) was used as a positive control in all screening assays. Following initial screening a total of 30 independent cell lines (clones) arising from all fusions were selected, seven of which were taken forward for further characterisation.

O2 M2: Select any for development, which appear specific for either *P. ramorum* and *P. kernoviae*, or are *Phytophthora* genus specific.

Selected cell lines were weaned off media supplements and bulked into large volumes to aid purification. A single antibody batch could then be used for optimisation and characterisation studies. Antibodies were isotyped to determine the class of immunoglobulin, which helps to select the method of purification. Antibody classified, as IgG's are typically the most reliable in LFD and ELISA formats and were purified using Protein G affinity columns (GE Healthcare). IgM Mabs have a pentameric structure, are difficult to purify and can be unpredictable in LFD and ELISA applications. IgMs were purified by Ammonium Sulphate Precipitation techniques. Antibody concentration was determined using a spectrophotometer at 280nm.

P.ramorum species-specific Mabs

Fusions 405, 406 and 407 generated 14 possible *P. ramorum* specific antibodies. Mabs D050, D056, D062 and D064 were selected as having the greatest potential. Early cross-reactivity testing with soluble protein extracts (SPE) and artificially infected leaf material showed a high degree of *P. ramorum* recognition only. However, in later screens using different batches of material, it was found that little or no detection of other *P. ramorum* isolates was possible.

Repetition of this assay with a diverse selection of other *Phytophthora* sp. and un-related artificially infected fungal pathogens was conducted and confirmed that *P. ramorum* detection was limited.

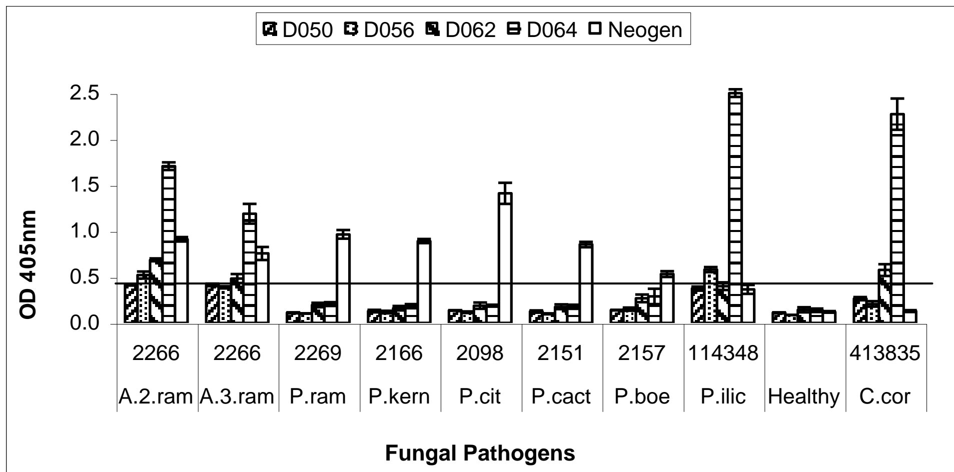


Figure 5a: A histogram of absorbance values following PTA –ELISA when testing *Phytophthora* sp SPE (*P.ramorum* A2/A3), artificially infected and healthy leaf material using putative *Phytophthora* species specific Mabs.

Note: Stdev from 3 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

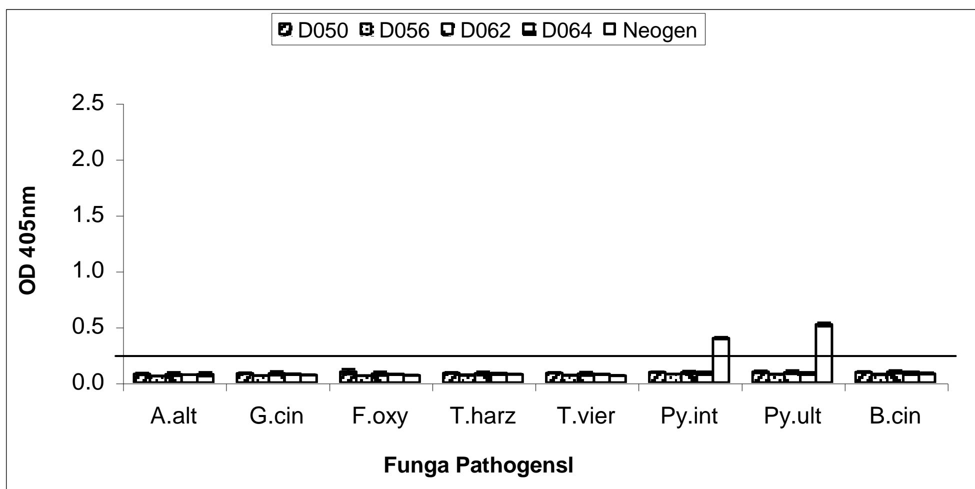


Figure 5b: A histogram of absorbance values following PTA –ELISA when testing non-phytophthora fungal isolates SPE (*P.ramorum* A2/A3), artificially infected and healthy leaf material using the putative *Phytophthora* species specific Mabs.

Note: Stdev from 3 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

The specificity of these antibodies was investigated in more detail as it was proposed that these results might be a result of antibodies that had been selected to a particular life cycle stage. An investigation was carried out where a large number of Rhododendron leaves were artificially infected with *P. ramorum* and *P. kernoviae,* in addition *P. ilicis* was infected onto Holly leaves. A time course study was conducted with one-day intervals from day three, up to a total of 14-days. Each day the samples were removed and frozen, these samples were then tested using PTA-ELISA with the panel of putative *P. ramorum* genus-specific antibodies (Figure 6a) and the *Phytophthora* genus specific antibodies (Figure 6b) D036, D037 and D051. The Neogen Mab was used as an assay control.

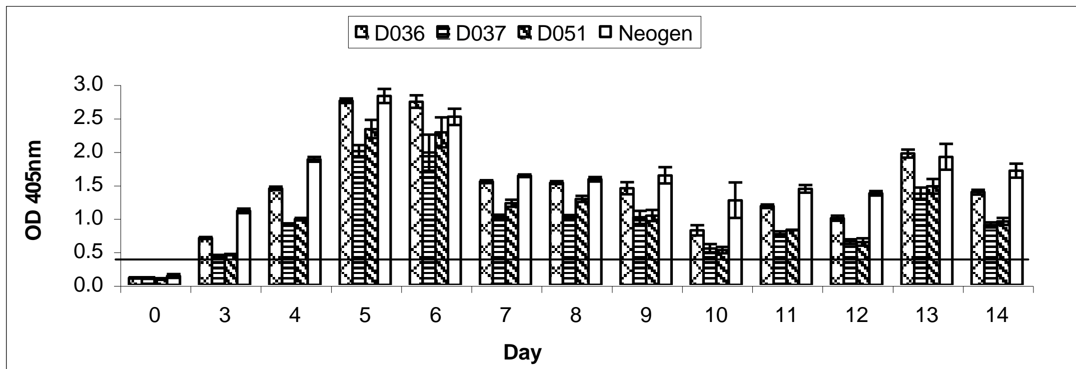


Figure 6a: A histogram of absorbance values following PTA –ELISA when testing *P.ramorum* infected leaf material daily over a 14-day period using *Phytophthora* genus specific Mabs.

Note: Stdev from 3 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

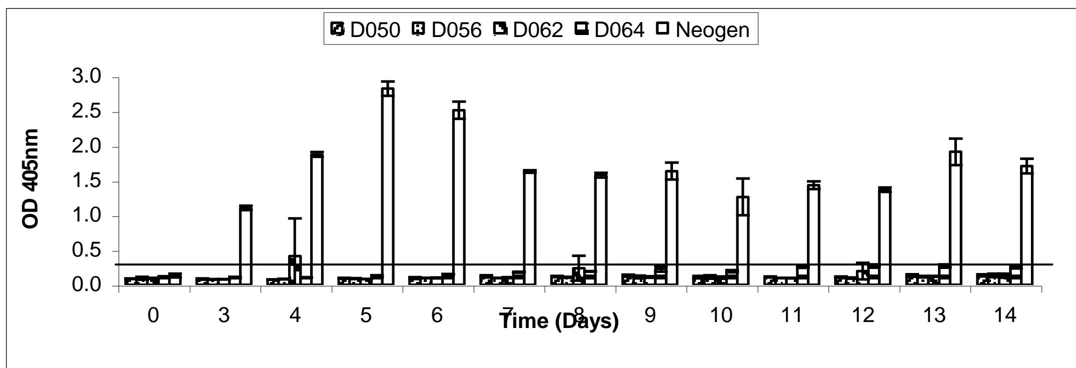


Figure 6b: A histogram of absorbance values following PTA –ELISA when testing *P.ramorum* infected leaf material daily over a 14-day period using putative *Phytophthora* species specific Mabs

Note: Stdev from 3 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

To investigate if these putative species-specific antibodies could be recognising a life stage (e.g. chlamydospores), further studies were conducted. The leaf study was repeated with only *P. ramorum* infected leaves but grown in both light and dark conditions to encourage the pathogen to produce different structures (e.g. the presence of chlamydospores was confirmed in the infected leaves by microscopic examination of washings from infected leaf material). Results showed that the potentially specific antibodies failed to recognise any of the infected material in either light or dark conditions, the genus specific antibodies showed a similar pattern of recognition as the Neogen Mab.

These antibodies, although initially selected as having species-specific profiles, were discounted from future assay evaluations. It was concluded that they were specific to a protein present in the initial extracts used for immunisation; an observation confirmed by Western blot analysis using *P. ramorum* infected leaf material and the immunogen (Figure 7).

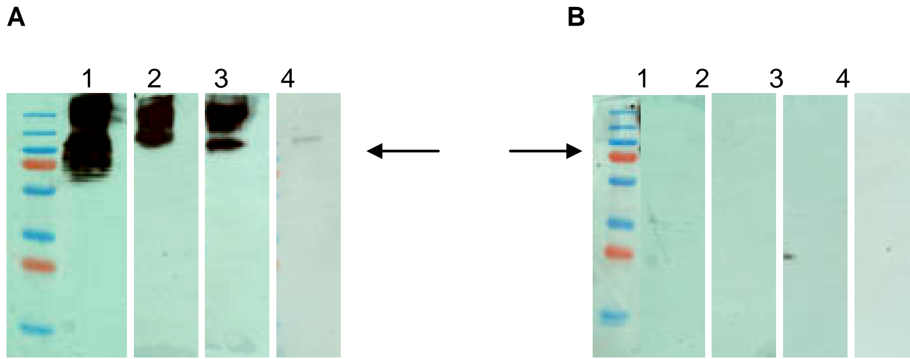


Figure 7: A montage of photographs of western blots of proteins from (A) the immunogen and (B) protein extracted from whole infected leaf samples, separated using SDS PAGE. The blots were probed with (1) Mab-D050 (2) Mab-D056, (3) Mab-D062 and (4) Mab D064. Proteins of sizes 72 - 100kDA are highlighted in A and B respectively.

P. kernoviae species specific Mabs

Fusion 414 generated 8 possible *P. kernoviae* specific antibodies. Early screening initially showed that these antibodies had a *P. kernoviae* specificicity (figure 8A), however following 1st cloning extensive ELISA testing against many isolates of *P. kernoviae* and *P. ramorum* soluble protein extracts (SPE), agar plate washings and artificially infected leaf material showed that little or no detection of other *P. kernoviae* isolates was possible (figure 8B).

These antibodies were discounted from future assay evaluations. It was concluded that they were again specific to a protein present in the initial extracts used for immunisation

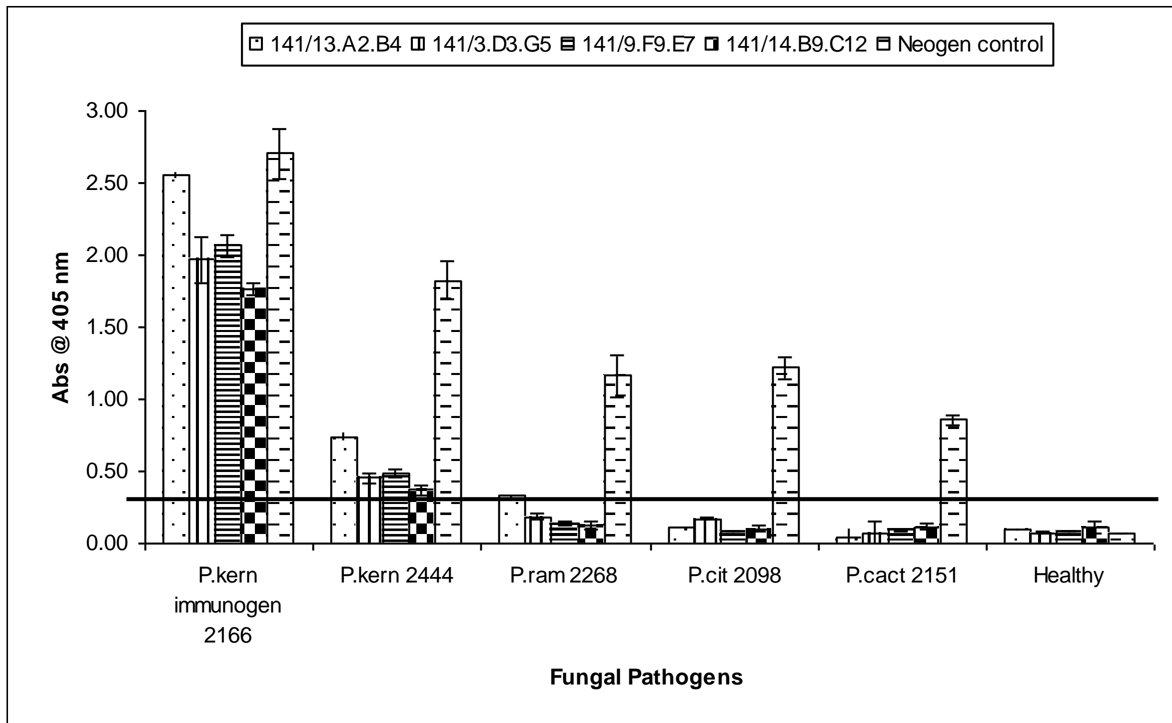


Figure 8a: A histogram of absorbance values following PTA –ELISA when testing *Phytophthora* sp SPE (*P.kernoviae* 2166), artificially infected and healthy leaf material using putative *P.kernoviae* specific Mabs.
Note: Stdev from 2 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

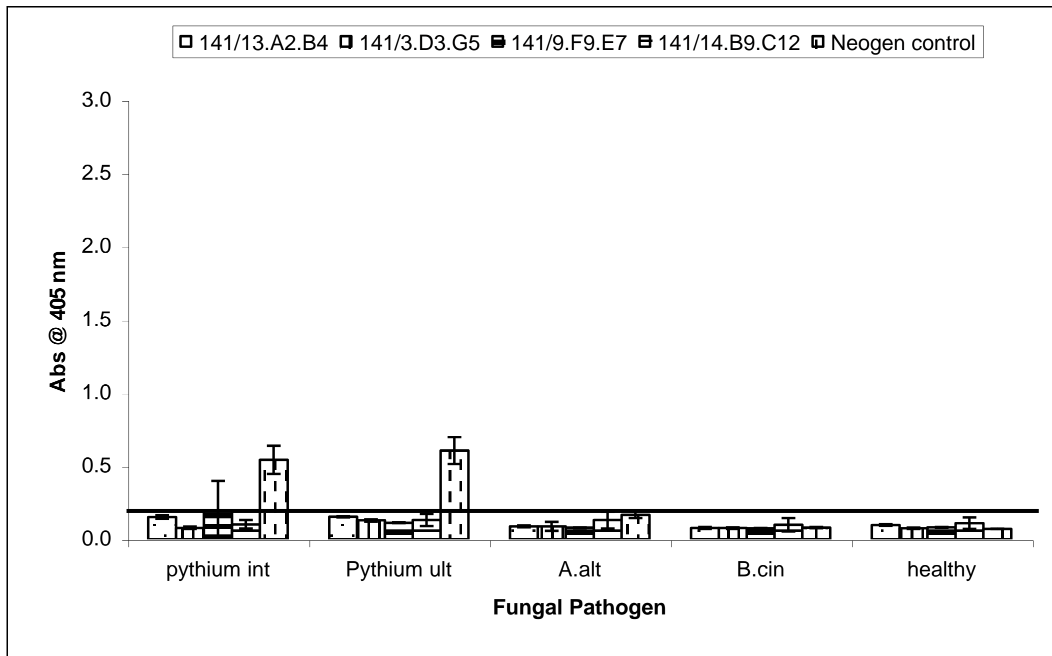


Figure 8b: A histogram of absorbance values following PTA –ELISA when testing non-phytophthora fungal isolates SPE, artificially infected and healthy leaf material using the putative *P.kernoviae* specific Mabs.
Note: Stdev from 2 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

Phytophthora genus-specific Mabs

Fusion 405 generated 16 *Phytophthora* genus specific antibodies. The monoclonals D036 D037 and D051 were investigated in more detail. ELISA testing of these antibodies showed that they had specificity profiles comparable with the existing genus specific Mab (Neogen) when tested against Soluble Protein Extracts (SPE) of *Phytophthora* isolates (figure 9A).

The selected Mabs were tested against a diverse selection of fungal pathogens. The results showed all the monoclonal antibodies gave good recognition of the positive material but the detection of *P.illis* was not achieved in this test unlike previous assays. All of the selected antibodies cross-reacted with *Pythium sp* in ELISA screening, but the OD reached was less than the existing genus specific Mab (Neogen) (Figure 9B).

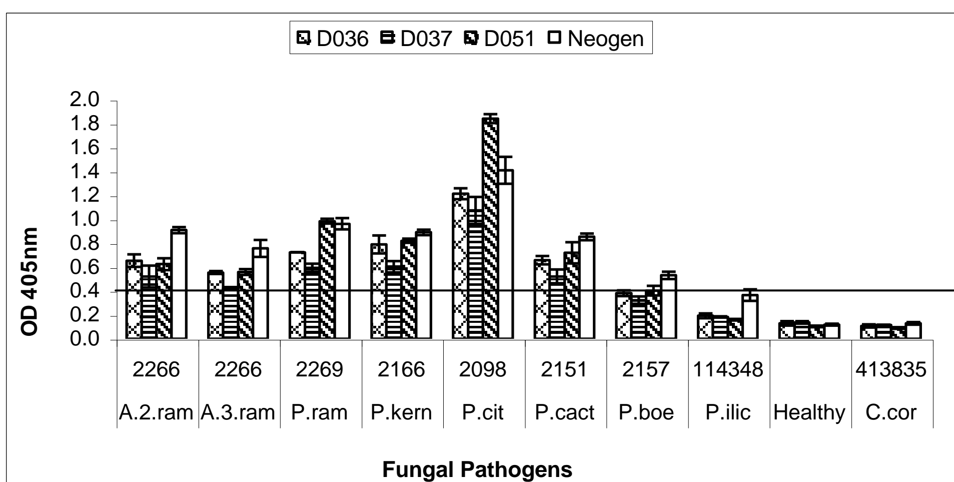


Figure 9a: A histogram of absorbance values following PTA –ELISA when testing *Phytophthora* sp SPE (*P.ramorum* A2/A3), artificially infected and healthy leaf material using genus specific Mabs.
Note: Stdev from 3 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

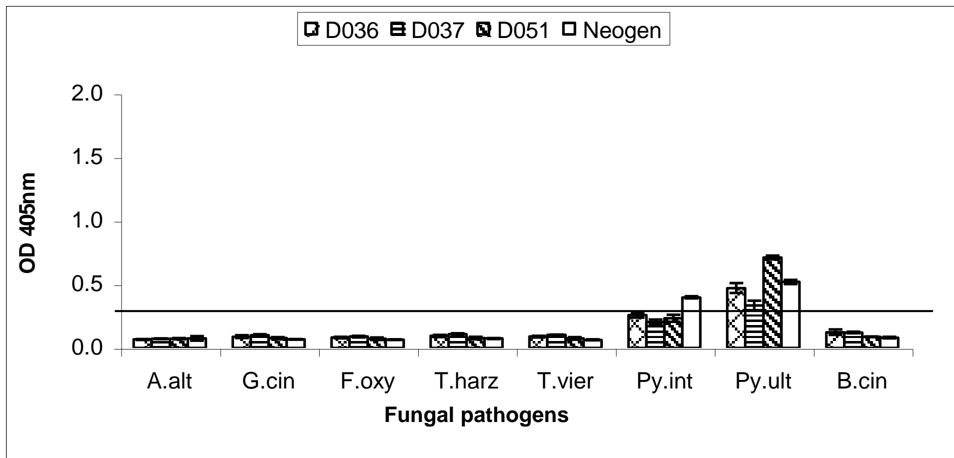


Figure 9b: A histogram of absorbance values following PTA –ELISA when testing unrelated fungal pathogen SPE (*P.ramorum* A2/A3), artificially infected and healthy leaf material using the *Phytophthora* genus specific Mabs.
Note: Stdev from 3 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

Following ELISA testing, all three of the selected Mabs gave similar results. Western blotting (Laemmli 1980) was then used to further characterise these reagents using protein extracted from whole infected leaves and soluble protein extracts. Results (Figure 10) show that a larger target (72kDa) was recognised in protein extracted from infected leaf material than in the immunogen preparation (60kDa) Figure 10. This difference is likely to be because the antigen is processed differently by the pathogen when infecting plant material than when in culture.

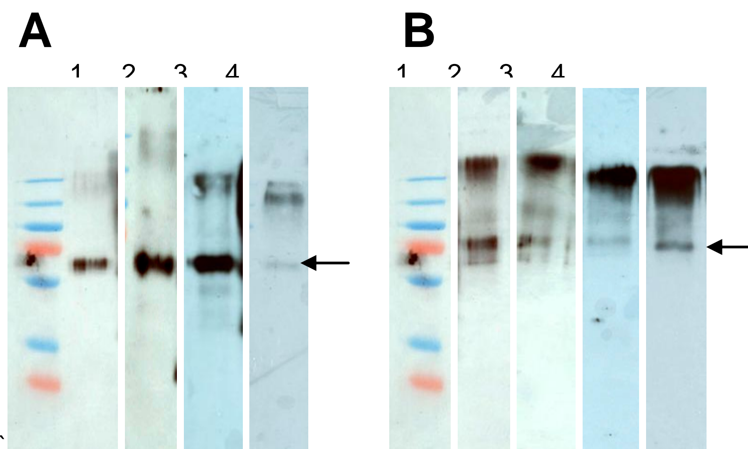


Figure 10: A montage of photographs of western blots of proteins from (A) the immunogen and (B) protein extracted from whole infected leaf samples, separated using SDS PAGE. The blots were probed with (1) Neogen (2) Mab-D036, (3) Mab-D037 and (4) Mab D051. Proteins of sizes 60kDA and 72kDA are highlighted in A and B respectively.

The three *Phytophthora* genus specific antibodies were taken forward for further optimisation and characterisation studies.

O2 M3: Fully optimise any selected antibodies

An affinity and cross reactivity study of each of the genus specific antibodies was performed.

(i) ELISA

Validation ELISAs were performed using purified antibodies. The validation assay comprised of 12 replicates of each of the following: 8 x unrelated 'negative' SPE isolates, 6 x infected leaf material (*Phytophthora sp.*) and healthy controls.

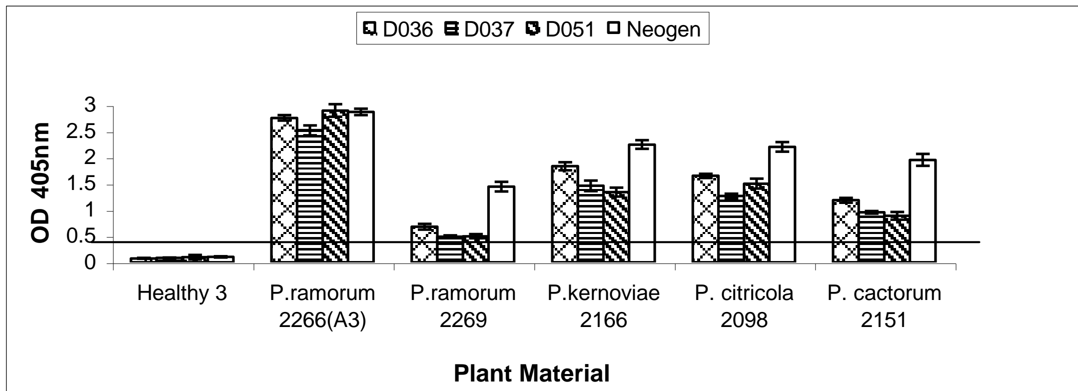


Figure 11a: A histogram of absorbance values following PTA –ELISA when testing *Phytophthora* sp infected plant material using the *Phytophthora* genus specific Mabs.
Note: Stdev from 12 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

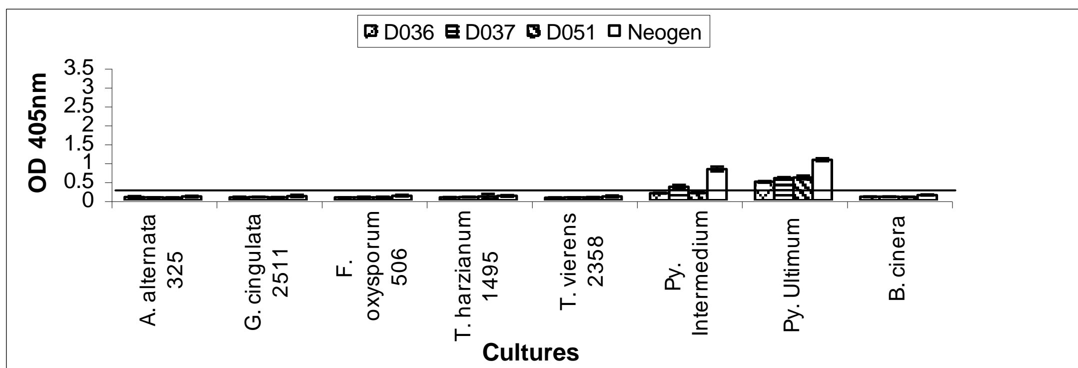


Figure 11b: A histogram of absorbance values following PTA –ELISA when testing unrelated non-phytophthora infected plant material using the *Phytophthora* genus specific Mabs.
Note: Stdev from 12 replicates and positive assay threshold value indicated by horizontal line (X2 negative control)

Antibody D036 detected all the *Phytophthora* species tested and did not cross-react with any of the unrelated fungal pathogens, with the exception of *Pythium sp*. The degree of cross-reaction was less than that observed with the other available genus Mab's (Neogen).

(ii) Antigen characterisation

The structure of the epitope recognised by the selected Mabs (D036, D037, D051) was compared using a number of techniques: (a) Heat treatment of the antigen, which destroys the secondary structure of pure protein (b) Periodate, which cleaves carbohydrate residues from glycoproteins and (c) Protease which hydrolyses peptide bonds in protein.

a) Heat - *P. ramorum* and *P. kernoviae* soluble protein extracts were heat-treated at 65, 75 or 100°C for 5 minutes or autoclaved at 121°C for 15 minutes. A negative control sample was incubated at room temperature. Following treatment all the proteins were used to coat plates for ELISA screening. The results (Figure 12a) showed that only autoclaving reduced the binding efficiency of any of the antibodies.

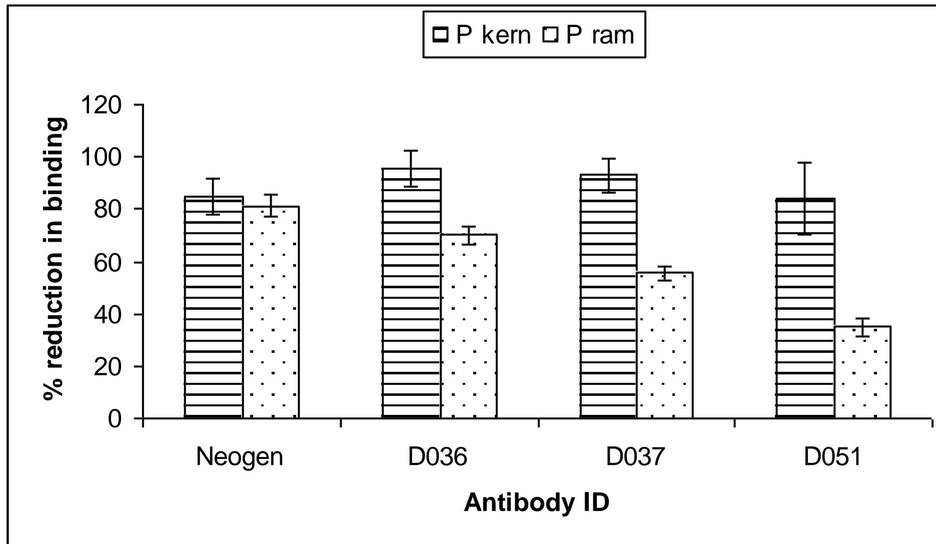


Figure 12a: A histogram showing the percentage of reduction in binding of antibody by PTA –ELISA following heat treatment (121°C) *of P.ramorum* and *P.kernoviae* antigens using *Phytopthora* genus specific Mabs.
Note: The mean of 12 replicates is expressed as a % reduction of binding in relation to untreated control.

b) Periodate – Antigen sensitivity to sodium periodate treatment was determined by exposing *P. ramorum* and *P. kernoviae* coated ELISA plates to 20mM sodium periodate in acetate buffer for various times over an 18hour period. Negative controls were treated with acetate buffer only. After treatment the Mabs were added and the PTA-ELISA completed. The results (Figure 12b) show that antibody binding to the *P. ramorum* antigen was reduced to 30-60% of the control after 30 minutes; D051 was most significantly affected by the treatment whilst the Neogen antibody the least. The effect of periodate reached a plateau of 11-20% after 7 hours.

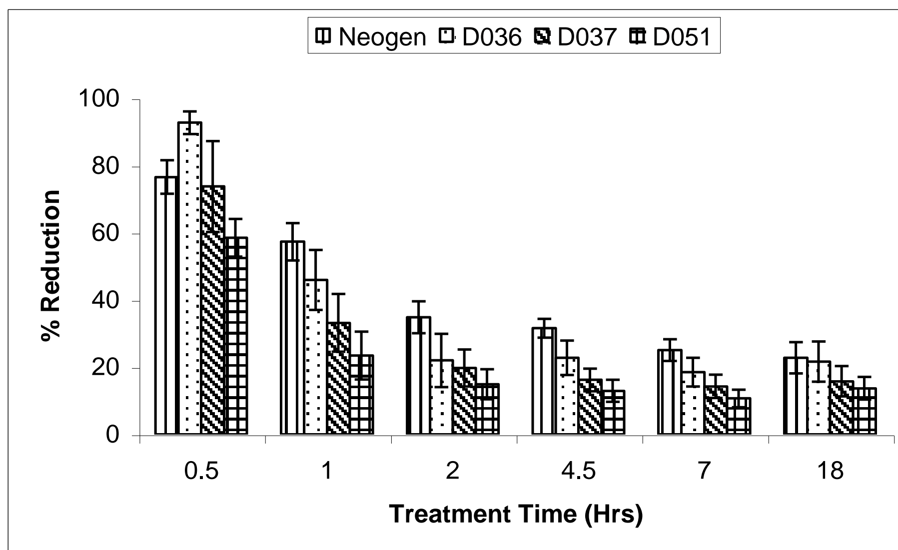


Figure 12b: A histogram showing the effect of time on the percentage of reduction in antibody binding by PTA –ELISA following periodate treatment *of P.kernoviae* antigens using *Phytopthora* genus specific Mabs.
Note: The mean of 12 replicates is expressed as a % reduction of binding in relation to untreated control

The results (Figure 12c) for periodate treatment on the binding of the antibodies to *P. kernoviae* SPE showed a similar trend, with regards to the length of treatment but reduction in binding was initially less pronounced.

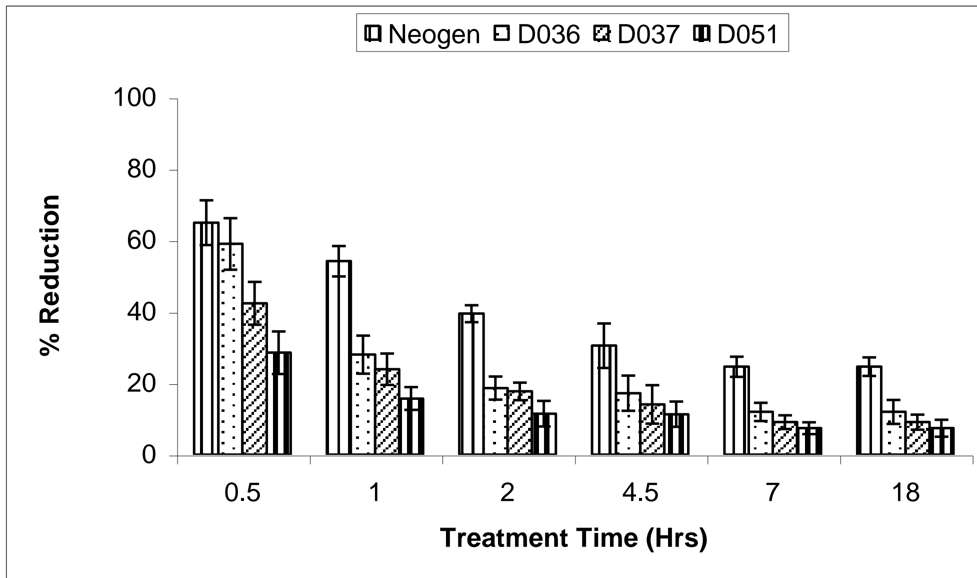


Figure 12c: A histogram showing the effect of time on the percentage of reduction in antibody binding by PTA – ELISA following periodate treatment *of P.ramorum* antigens using *Phytopthora* genus specific Mabs.
Note: The mean of 12 replicates is expressed as a % reduction of binding in relation to untreated control

c) Protease – Antigen sensitivity to protease digestion was determined by incubating *P. ramorum* and *P. kernoviae* coated ELISA plates with 0.25units of protease/well (in PBS) for 4 hours at 37°C, the negative controls contained PBS only. After treatment, the Mabs were added and the PTA-ELISA completed following the standard protocol. The results (Figure 12d) showed that reduction in binding occurred with all the antibodies.

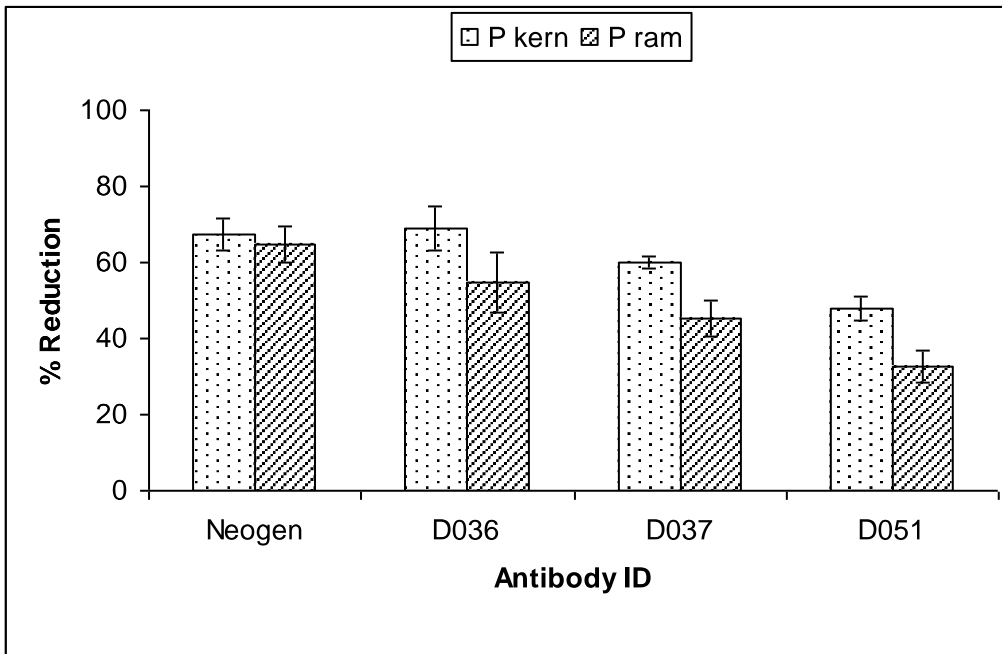


Figure 12d: A histogram showing the percentage reduction in binding by PTA-ELISA following protease treatment of *P.ramorum* and *P kernoviae* antigens using *Phytopthora* genus specific Mabs.
Note: The mean of 12 replicates is expressed as a % reduction of binding in relation to untreated control

Reduction in binding following protease digestion shows that all the antibodies bind at least in part to protein epitopes. The sensitivity of the epitopes to periodate treatment indicates that antibody is also binding to glycoprotein molecules. Boiling the antigens for 5 minutes had no effect on antibody binding and autoclaving only partially reduced binding, confirming that the epitopes do not incorporate complex protein secondary structures. Taken together these data indicate the epitope for these Mab's is most likely at the junction between protein and carbohydrate of a glycoprotein.

It is interesting to note that the epitopes on the antigens from the two *Phytophthora* species appear to react differently. The epitope from *P. ramorum* is more sensitive to autoclaving; periodate treatment and protease digestion than that from *P. kernoviae*. It is also apparent that the epitope for each antibody has a different profile of sensitivity. D036 and D037 appear to be very similar to each other; all three treatments adversely affect binding of D051, the Neogen Mab is less so. This confirms that although each of the antibodies has similar species specificity, they do not recognise the same epitope.

(iii) Antibody Sequencing

Since the antibody's selected had similar specificity the variable light chains were PCR amplified and sequenced to identify if the monoclonal antibodies were different. If the antibodies differed they would be included in all combinations in LFD's to empirically test for the best combination. If identical antibodies were found only one would be selected for LFD optimisation.

RNA was extracted from cells expressing antibodies D036, D037 and D051. This RNA was reverse transcribed to produce cDNA and PCR carried out using primers designed to amplify the variable light antibody chains. The primer sequences were obtained from Clackson et al (1). PCR fragments 332 bp long were obtained using the light chain PCR primers from all three RNA samples. The light chain PCR fragments were cloned into pGEMTeasy and sequenced using T7 / M13 Forward primers.

The variable region of the light chains from antibodies D036 and D037 were 99.6 % identical (1 base pair difference) and antibody D051 was 95 % identical to D036 and D037 (13 base pair differences). The DNA sequences were translated into protein sequence and realigned. The alignment (Figure 13) shows that antibodies D036 and D037 had 99% homology (1 amino acid difference) and antibodies D036 / D037 were 89% homologous to antibody D051 (10 amino acid differences).

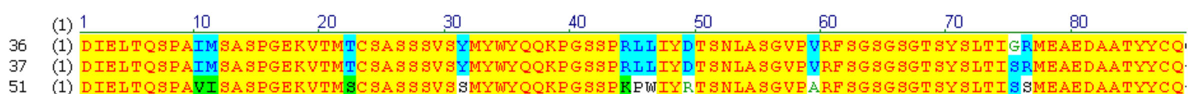


Figure 13. Alignment of the protein sequences for the variable light chains of *Phytophthora* genus specific Mabs. Identical amino acids are highlighted in yellow, weakly similiar amino acids are highlighted in green, non-similiar amino acids in white and conserved amino acids are highlighted in blue.

Based on sequencing of the light chains, antibodies D036 and D037 are similar but not identical and antibody D051 is different, thus all were taken forward for LFD optimisation.

O3 M1 Develop Lateral Flow Devices using any *P.ramorum* or *P.kernoviae* species-specific antibodies generated.

No species-specific Mabs were generated, however the genus specific Mabs were optimised in an LFD format.

O3 M2 Develop a genus specific LFD, to replace existing version, complete a full comparison and validation.

And

O4 M1 Use genus specific antibodies to develop *Phytophthora* genus specific LFD

Antibodies D036, D037 and D051 were assessed in LFD format. The Mabs were used both as the target line sprayed onto the membrane surface and also passively bound to latex beads. Various concentrations of each were tested during initial testing and in total 80 different combinations were investigated (Table 3). Combination H

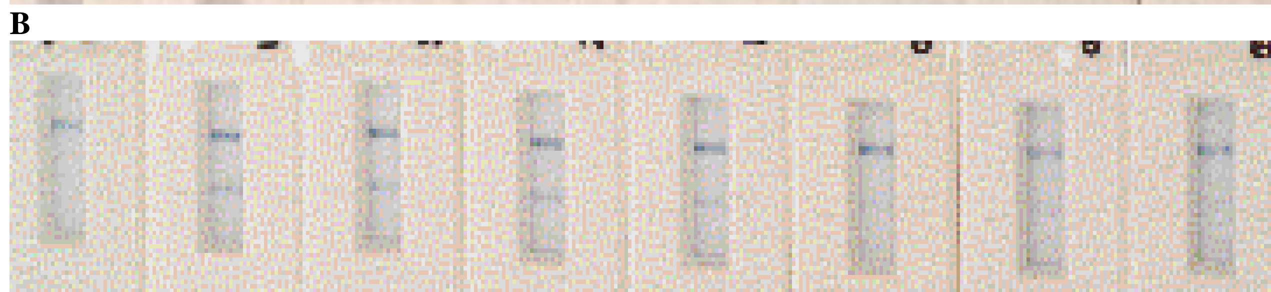
(Membrane: Mab D036, Latex: Mab D037) was chosen and used to produce prototype kits. This combination was selected as it produced uniform visual detection of *Phytophthora sp* target extracts, consistent line intensity and low background.

The specificity of the prototype device was compared with the existing commercial LFD by testing a range of plant pathogens i.e. *Phytophthora* genus-specific antigen, *Rhizoctonia* genus-specific antigen*, Erwinia amylovora*, and *Ralstonia solanacearum* bacterial suspensions. The results (Figure 14) show that a positive result was generated within 5 minutes of application of sap extracted from all *Phytophthora* sp infected samples, no false negatives were detected. Negative controls consisted of non-*Phytophthora* fungal pathogens, healthy leaf material and extraction buffer alone. No unexpected cross-reactions either with non-*Phytophthora* pathogens or any other negative control was observed.

Figure 14: Photographs of prototype LFDs (1-7 left to right) following testing of a range of fungal and other plant pathogens. 1 = Healthy leaf (negative control), 2 = Generic *Rhizoctonia sp*. positive antigen (Neogen) at 5ug ml^{-1}, 3 = Generic *Phytophthora sp*. positive antigen (Neogen) at 5ug ml^{-1}, 4 = Generic *Phytophthora sp*. positive antigen (Neogen) at 0.5ug ml^{-1}, 5 = *Erwinia amylovora* bacterial suspension at $1x10^7$CFU , 6 = *Ralstonia solanacearum* bacterial suspension at $1x10^7$CFU , 7 = Buffer C (negative control)

A

B



Negative control	$10ugml^{-1}$	$5ugml^{-1}$	$2.5ugml^{-1}$	$1.25ugml^{-1}$	$0.5ugml^{-1}$	$0.25ugml^{-1}$	$0.125ugml^{-}$

Figure 15: Photographs showing the serial titration of Neogen *Phytophthora sp.* positive antigen (10-0.125 ug ml^{-1}). (A) prototype LFDs (B) Neogen P*hytophthora* LFDs.
Note: Negative control is *Rhizoctonia* positive control at $5ugml^{-1}$

The sensitivity of the prototype device was then compared to the existing commercial LFD by testing a dilution series of generic *Phytophthora sp.* positive antigen from Neogen. The results show (Figure 15) that the prototype devices and the commercially available LFD's give a strong positive result after 5 minutes at a concentration of 0.5 $ugml^{-1}$ *Phytopthora* positive control antigen. A weaker line is still evident at 0.25 $ugml^{-1}$.

A test was performed comparing the prototype LFD with the commercially available LFD using 107 field infected leaf samples sent to the CSL Plant Health Diagnostic laboratory. The validation results (Figure 16) show that the prototype device has a 96% sensitivity {A / A+C}, and 100% specificity {B / B+D}. The positive predictive value of the new assay was 100% {A / A+B} and the negative predictive value was 91% {C / C+D}.

		Neogen LFD Devices		
		+	-	Total
LFD Prototypes	+	72 A	B 0	72
	-	C 3	D 32	35
	Total	75	32	107

Figure 16: Comparison of *Phytophthora* sp CSL LFD prototypes with *Phytophthora* sp Neogen LFD devices on intercepted PHSI Diagnostic Plant material, illustrating diagnostic sensitivity ($\frac{A}{A+C}$) and specificity ($\frac{D}{D+B}$) in each case. Diagnostic sensitivity = 96% and Diagnostic specificity = 100%

Table 3: Table showing the antibody combinations tried to optimise the *Phytopthora* LFD

Protoptype ID	Membrane		Latex		Result (P/N)
	Antibody ID	Concentration (ug/ml)	Antibody ID	Concentration (ug/ml)	
A	D037	2.0	D037	2.0	P
B			D051	1.0	N
C				2.0	N
D				3.0	N
E	D036	3.0mg/ml	D036	2.0	N
F				1.0	N
G				3.0	Y
H			D037	1.0	P
I				2.0	P
J			D051	1.0	N
K				2.0	N
L				3.0	N
M	D051	1.0mg/ml	D036	1.0	N
N				2.0	N
O				3.0	N
P			D037	1.0	N
Q				2.0	N
R			D051	1.0	N
S				2.0	N
T				3.0	N
U			D036	1.0	N
V	D051	2.0mg/ml	D036	2.0	N
W				3.0	N
X			D037	1.0	P
Y				2.0	P
Z			D051	1.0	N
Z1				2.0	N
Z2				3.0	N

O3 M3 Develop an integrated LFD/Molecular approach for field detection and increased specificity, to go from genus to species specificity.

Real-time PCR assays have been previously developed and validated for the detection of both *P. ramorum* and *P. kernoviae*, these assays are in use, in conjunction with conventional DNA extraction methods, and in parallel with isolation techniques, for the routine detection of *P. ramorum* and *P. kernoviae* in plant material at CSL. An investigation was performed to see if these assays could be used to detect *P. ramorum* and *P. kernoviae* in a sample which has given a positive result by *Phytophthora* sp. LFD, by directly testing the LFD membrane in real-time PCR reactions.

A method was developed for the detection of DNA on the membranes of lateral flow devices. After the sample was run, a section of the membrane was removed and added directly to a real-time PCR (TaqMan) reaction containing primers and probe specific to the target of interest. The DNA was found to be stable on the device; real-time PCR can be carried out immediately or after a period of storage at room temperature.

O4 M3 Extract DNA from positive lines & O4 M4 Develop diagnostic methods using real time PCR to confirm species in the laboratory

Samples of *P. ramorum* and *P. laterals* mycelium from cultures grown on carrot piece agar (CPA) were ground in bottles of lateral flow device (LFD) Buffer C (Forsite Diagnostics) by shaking for approximately 30 seconds, then approximately 60µl of homogenate was run on *Phytophthora sp.* lateral flow devices. Control and test lines developed on both devices within 5 minutes. The membranes were then removed from the plastic housing and strips of membrane incorporating the test lines were cut out using a scalpel blade. Each membrane strip was cut in half, and each piece was placed in a separate well of a 96-well plate containing 25 all master-mix for real-time PCR (TaqMan) for P. ramorum (1 x PCR buffer (50 mom KCl, 10 mom Tris-HCl, 0.001% gelatin), 0.025 U/all Hot Taq, 0.2 mom each dNTP, 5.5 mom MgCl2, 1 x ROX passive reference dye, 300 nM each *P. ramorum* primer and 100 nM P. ramorum probe). Real-time PCR was carried out on an ABI 7900HT, using generic cycling conditions of 10 min at 95°C followed by 40 2-step cycles of 15 s at 95°C and 1 min at 60°C.

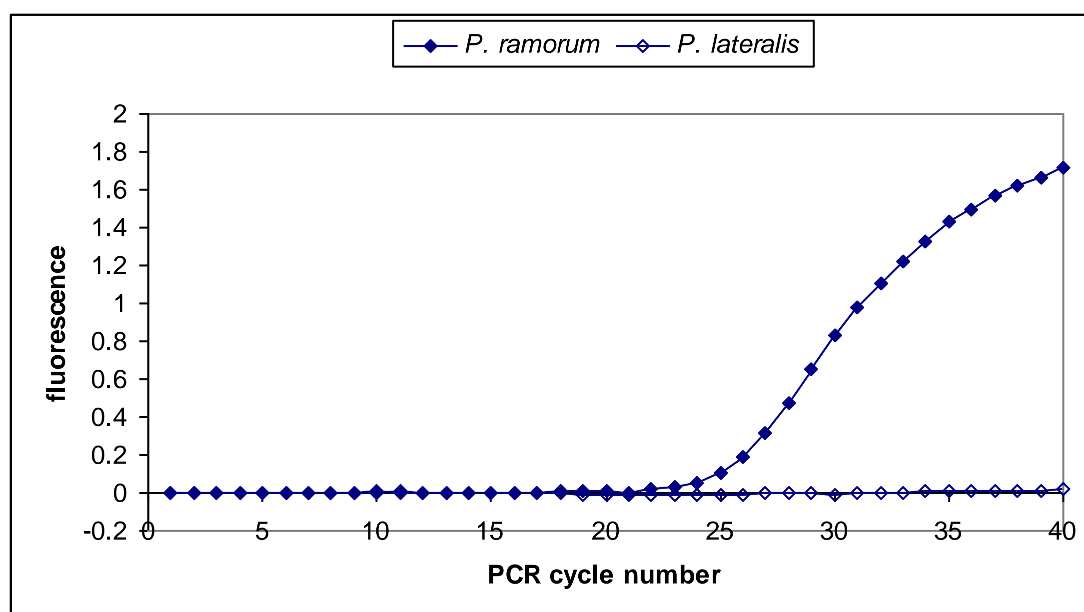


Figure 17: Amplification plots for TaqMan real-time PCR testing of membranes taken from lateral flow devices run with samples of *P. ramorum* and *P. laterals*.

The results (figure 17) show that although the LFD was positive for both samples, DNA from the pathogen within the device could be used as a template in a real time reaction to give specific detection of P ramorum. Further work needs to be performed on stability of the DNA present within the membrane to investigate if the positive devices could be returned to the laboratory for species discrimination using either real-time PCR (TaqMan) or sequencing. This could potentially be used as an alternative to returning leaf samples for DNA extraction, potentially speeding up the testing and reducing sampling errors (i.e. the exact same sample that gave the LFD result would be used for the real-time PCR test). In addition it could form the basis of an extraction system for more simplified/specific molecular tests as investigated in projects PH0305 and PH0424 for use in the field.

O4 M2 Use LFD to identify *Phytophthora* ramorum infection in the field and send to laboratory for confirmation

The performance of the LFD extraction / TaqMan method for the detection of *P. ramorum* and *P. kernoviae* in field samples was compared with that of the laboratory methods currently in use. On receipt in the laboratory, field samples were split into two replicate samples; one half was tested for *P. ramorum* and *P. kernoviae* in the diagnostic laboratory, while the other half was shaken in a bottle containing LFD Buffer C and ball bearings and applied to a *Phytophthora* sp. LFD. Segments taken from the LFD membrane were added to TaqMan reactions

for *P. ramorum*, *P. kernoviae* and plant DNA (cytochrome oxidase, or COX). The testing method used in the diagnostic laboratory was dependent on the host species: in general, samples of rhododendron were tested directly by real-time PCR using a modified KingFisher DNA extraction from the plant material followed by testing for *P. ramorum*, *P. kernoviae* and COX by TaqMan. Non-rhododendron hosts were tested by plating on semi-selective media and assessed for growth of *P. ramorum* or *P. kernoviae* as determined by morphological examination. Some rhododendron samples were tested by plating instead of TaqMan for logistical reasons (such as sample size), and all rhododendron samples for which conventional DNA extraction was considered to have failed (negative COX TaqMan result: 15 samples) or been inefficient (high COX Ct: 4 samples), or which gave ambiguous results for *P. ramorum* or *P. kernoviae* by TaqMan (Ct value >36 for *P. ramorum* or >30 for *P. kernoviae*, 5 samples) were subsequently tested by plating to obtain a result.

Positive LFD results were obtained for 149 out of 202 samples received. Neither *P. ramorum* nor *P. kernoviae* was detected by TaqMan real-time PCR on any of the 53 devices that were negative for *Phytophthora* by LFD. DNA was successfully extracted from 146 of the 149 LFD-positive samples (98.0%), as indicated by a positive COX TaqMan result or a positive result for *P. ramorum* or *P. kernoviae*. Figure 18 shows how the results for these samples compare to the results obtained in the diagnostic laboratory using standard methods. The method of testing positive *Phytophthora* LFDs for *P. ramorum* and *P. kernoviae* by direct TaqMan PCR from the LFD membrane was found to accurately predict the results of routine testing in the diagnostic laboratory, with a positive predictive value (A/A+B) of 92.6% and a negative predictive value (D/C+D) of 92.1%.

		Diagnostic laboratory result		
LFD extraction + TaqMan result		+	-	Total
	+	100 (A)	8 (B)	108
	-	3 (C)	35 (D)	38
	Total	103	43	146

Figure 18: Comparison of LFD DNA extraction (followed by TaqMan real-time PCR) and routine laboratory testing for the detection of *P. ramorum* and *P. kernoviae* in field samples.

Policy interpretation, impacts and benefits

There are a number of benefits coming from this work, despite the fact that species-specific antibodies and therefore lateral flow devices have not been developed.

- Currently *Phytophthora* LFD's are produced with a commercial antibody. There are no guarantees with regards to the long-term supply of this reagent for this purpose; it is also not known if there is a finite supply of the reagent. The new reagents produced in this work give almost identical results in both ELISA and LFD to the existing reagents used, these are Monoclonal antibodies, as such they are produced in immortalised cell culture, thus this reagent will be available indefinitely for the production of LFDs for the PHSI.

- Although not a primary goal of this work, the supply of the new reagents under existing supply agreements with Forsite Diagnostics would result in the production of a kit at least ¼ of the price of the currently uesd kits. This would undoubtedly have an impact on the number of samples that could be tested in the field by the PHSI in future work with *Phytophthora*.
- The work on testing the LFD membrane in order to allow speciation of *Phytophthora* present was highly successful, this could have a number of benefits. Firstly, if a positive LFD is achieved a second sample is not required to perform the speciation; this should reduce errors in the testing system resulting from uneven distribution of the pathogen and subsequent sub sampling since the exact same sample is tested using both methods. In addition the LFD could be returned to the laboratory for confirmatory testing rather than leaf samples, thus less infectious plant material would be circulating in the postal system. In addition the tissue sampling and extraction of nucleic acid from the sample is, in effect completed in the field, thus sample processing in the laboratory would be reduced; further reducing costs and speeding up the service from the laboratory.

Recommendations

Several recommendations could be made at this stage:

- The prototype LFDs that have been produced do not under laboratory conditions give exactly the same results as the currently used LFDs. As might be expected these currently used LFDs do not give the same results as laboratory testing – though in previous comparative evaluations are suitable for achieving the task for which they are being used. With this in mind I would recommend that the prototype LFDs are subjected to a validation study by inspectors on real samples, compared with laboratory testing such that the same evaluation regarding the devices being fit for purpose can be made. **This study is underway and is being coordinated by the CSL diagnostics team and the PHSI. See Annex 2.**

- The confirmation of species using molecular tools from positive LFDs was also very successful, in the validation study carried out there were very good positive predictive values using this approach, as compared to the full laboratory service incorporating all methods possible. The LFDs were run in this study in the laboratory from samples sent to the laboratory by the PHSI. It is necessary to grind the sample with clear adherence to a recommended protocol (more important than with conventional LFD) and to allow the device to dry before testing to ensure the stability of the DNA. With this in mind a validation study is necessary in which samples are run in the field by PHSI and these are returned to the laboratory along with the samples such that the recommended grinding protocols and dry transport of the device can be evaluated. **This study is underway and is being coordinated by the CSL diagnostics team and the PHSI. See Annex 1 for additional work investigating the shaking approaches on LFD DNA extraction.**

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

1. Clackson T, Hoogenboom HR, Griffiths AD and Winter G, Making Antibody Fragments Using Phage Display Libraries. Nature 352 624-628 (1991).

2. Laemmli U.K. (1980) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature; 227; 15. 680-685.