DNAiTECH

Marlborough Research Center,
Grovetown, Blenheim
Marlborough, New Zealand 7202
+64 2142 9962 | +64 3579 5443
dnaitech@gmail.com

Plant and Food Research Limited
4th March 2024
THEME 5 S15 Robust detection of *Phytophthora agathidicida*: from laboratory to in-field beta testing

REPORT

The goals were to develop 1): the prototype hardware device for *Phytophthora agathidicida* in-field diagnostics 2): microfluidic chips with dried reagents for detecting phytophthora DNA 3): extractions of soil containing phytophthora DNA and 4): deliver a training workshop to demonstrate the prototype diagnostic tests.

MILESTONE PROTOTYPE DEVICE: DNAITECH GENERATION 3, VERSION 3 DEVICE.

Under this funded program we have developed the prototype device with integrated electronics and onboard rechargeable lithium-ion battery for in-field operation. This device has three diagnostic chip positions for the simultaneous running of 3 diagnostic chips. The three diagnostic chips are illuminated by blue LEDs at 460nm and the fluorescence of the LAMP-CRISPR products of a positive reaction are visualized through the green filter window of the instrument. The positive reactions can be captured by camera and but also are intense enough to be visualised by naked eye. Image below left shows the laser cut and assembled prototype instrument and, on the right, showing the instrument with the three filter/access windows removed and device with the 460nm internal illumination turned on.

MILESTONE DEVELOP MICROFLUIDIC CHIPS WITH FREEZE DRIED REAGENTS INCORPORATED FOR DETECTING PHYTOPHTHORA DNA, AND ALPHA TEST THE COMPLETE SYSTEM IN THE DNAITECH LABORATORY.

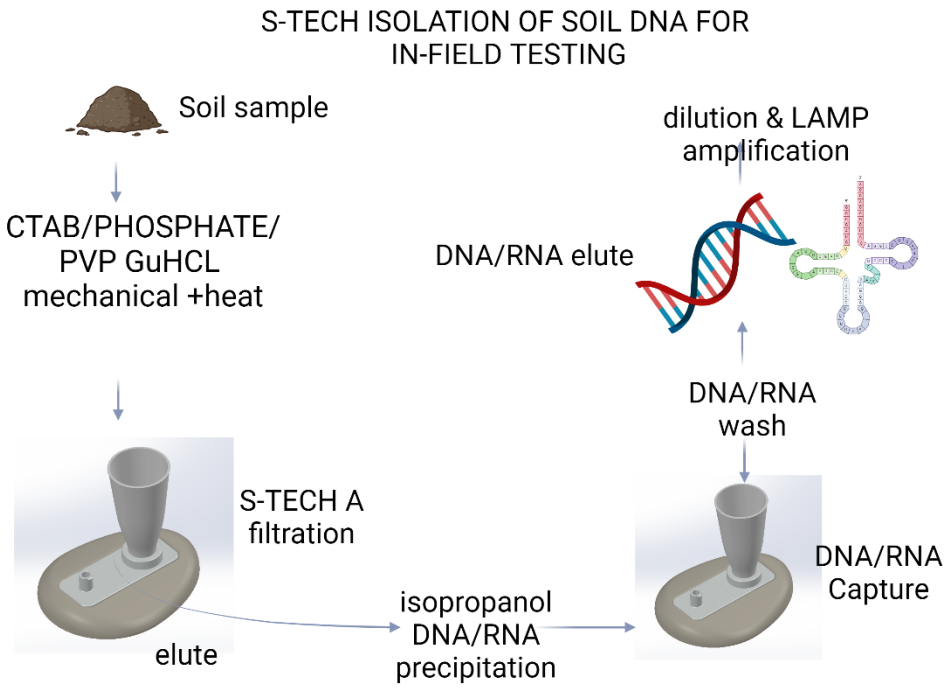
There were some technical issues found initially getting the *Phytophthora agathidicida* assay running on the chips, surprisingly predominantly with the genomic DNA rather than G-block DNA. This may relate to some structural secondary or tertiary structure in the genomic DNA that is not present in the very short G-block sequences. The phytophthora assay (Winkworth published assay) is the slowest of all our diagnostics LAMP assays for our range of targets, it required higher levels of the enzyme to coach it along on the chips. Optimising the LAMP time and temperature on the chips and controlling other parameters enabled successful running of both G-block and genomic DNA.

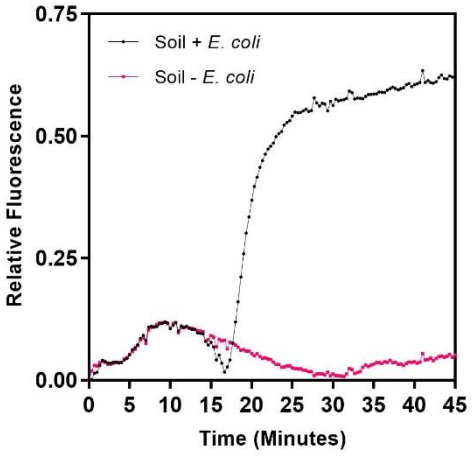
The images below show the results of a diagnostic chip with dried reagents imbedded into the chip, as required for delivery of point-of-care/in-field diagnostics. Image of the whole instrument with illuminated chip (left) and zoomed in image of the fluorescent chip (right).

The chip positions are as follows.
Position top left phytophthora DNA approximately 70,000 copies, dried reagents in chip.
Position top right phytophthora DNA approximately 70,000 copies, dried reagents in chip.
Position bottom right, G-block DNA approximately 105,000 copies, wet reagents flowed in.
Position bottom left; minus DNA control.

SOIL EXTRACTIONS.

DNAiTECH has developed a nucleic acid extraction technology (S-TECH) that enables soil and water extractions in the field. The technology uses a classical CTAB extraction chemistry (with minor modifications), the primary filter enables clearing the extracts of soil particles and debris and the second filter for capture of isolated nucleic acids on glass-fibres. The cleaning of the DNA/RNA occurs on the filter and the nucleic acids are eluted with water or TE buffer. The process is fully implemented using syringes, bypassing the requirement for laboratory equipment such as centrifuges and spin columns. The nucleic acids isolated from soils using this method are of high purity, sufficiently free of inhibitors such as humic acids, enabling inhibition free isothermal reactions. The soil protocol is summarised in the diagram below.



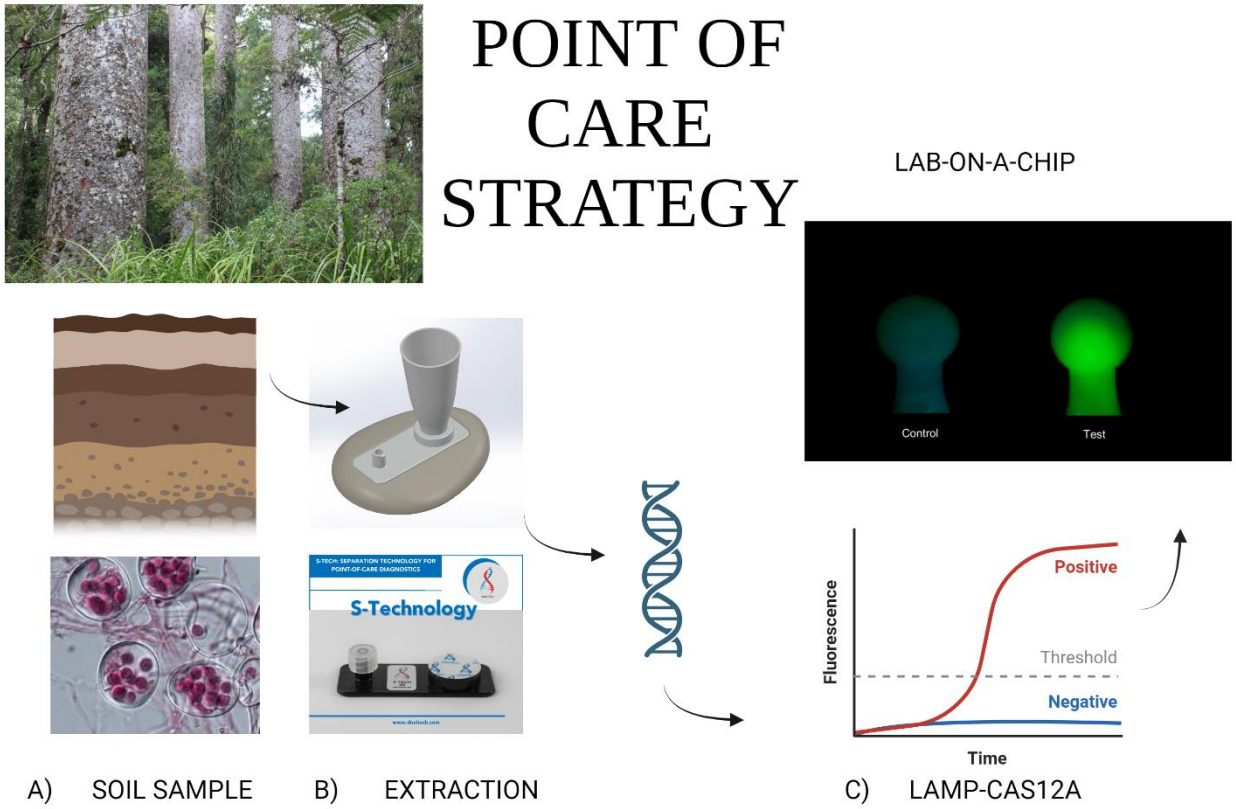


Our objective to extract soils in this project using soil samples spiked with phytophthora DNA was not achieved, because of the tight timeframes of this project. We had aimed to repeat what is shown (left) for soil samples spiked with *E coli* cells, except using phytophthora DNA instead of *E. coli*.

The S-TECH extractions has proven to be robust with samples that are notoriously difficult, for many applications, such as blood, faeces, biopsies, plant tissue etc.

PROJECT SUMMARY

This project achieved two significant goals, the development of the prototype in-field capable instrument and the demonstration of positive reactions on diagnostic chips using dried reagents imbedded in the chips. Positive reactions were achieved with both G-block and genomic phytophthora DNA. The combination of very tight time frames, staff with covid and some technical issues with the phytophthora assay on chips with genomic DNA resulted in us not being able to deliver the soil extractions and the anticipated workshop. However, this project does set the stage for the establishment of a viable point-of-care for phytophthora should funding be available to continue this work. Such a diagnostic test would consist of steps outlined in the below diagram.



NEXT STEPS FOR PROJECT CONTINUATION

Should this project progress to the next phase we would anticipate the following necessary development steps.

1) Phytophthora assay:

   We would recommend the development of a more robust and faster phytophthora assay. The Winkworth assay in our hands is relatively slow, in both tubes and diagnostic chips. An alternative assay would require the identification of suitable PAM sites for Cas12a DNA, that does not overlap the LAMP primers.

2) Sensitivity and Specificity testing:

Assays on chip are ideal for in-field testing because the reagents are prepackaged in dried form into the chips for enzyme stability and the user simply applies the sample, the chip is placed into the instrument/hardware for temperature control and illumination and the LAMP reaction proceeds on the chip within the hardware. The CRISPR reaction steps occurs at the completion of the LAMP reaction.

If a new phytophthora assay was utilised, it would be necessary to statistically validate the assay sensitivity, aiming to achieve positive LAMP reactions within 15-20 minutes, and ideally detecting down to 10-50 genomic equivalents of phytophthora DNA in the sample. The harnessing of CRISPR reactions readout to LAMP reactions has major benefits. Firstly, the CRISPR readout of the LAMP reaction is intensely fluorescent, it is visible to the eye and able to be captured by a smartphone camera. The other benefit is that when the PAM site for the CRISPR reaction is on the amplicon but not overlapping the LAMP primer regions, this eliminates nonspecific amplification, which can occur with LAMP alone. Thus LAMP-CRISPR has very high specificity, i.e. the absence of false positive reactions.

3) Extraction of DNA:

As was planned for this project, the development of a robust assay would then lead to the validation of chip assays using soils spiked with phytophthora DNA or organisms extracted with the S-TECH soil extraction. As we have already verified this with other organisms including *E. coli* we anticipate this to be relatively straight forward transition. This assumes that phytophthora is readily lysed using the classical CTAB/guanidine HCl extraction chemistry routinely used for soil extractions. However, this will need validation, some of the life cycle stages of phytophthora agathidicida which may be more or less susceptible to such extraction and adjustment of the extraction regime for this organism may be required.

4) Validation of a diagnostic Test.

Successful diagnostic testing of DNA/organism spiked soil samples would then lead to screening of the screening of real-world soil samples that have already been validated by traditional validated methods for the presence and absence of phytophthora. This is a necessary for validation of any new diagnostic test, benchmarking the new test against a standard assay for verification of the new tests sensitivity and specificity.

5) Commercial Test.

DNAiTECH is transitioning away from prototype chips and hardware to injection moulded commercial devices in 2024/25. If the in-field phytophthora project was to continue to be funded through the above development phases, a viable POC test could be available in 2025.