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FINAL REPORT - SRDC PROJECT UQ024

"DEVELOPMENT OF DNA BASED DIAGNOSTIC SYSTEMS FOR SUGARCANE PATHOGENS"

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APPENDICES

1.0 ABBREVIATIONS:

CP, viral coat protein
Cxc, *Clavibacter xyli* subsp. *cynodontis*
Cxx, *Clavibacter xyli* subsp. *xyli*
DNA, deoxyribonucleic acid
dsDNA, double-stranded DNA
ssDNA, single-stranded DNA
ELISA, enzyme-linked immunosorbent assay
EB-EIA (or EB-ELISA), evaporative-binding ELISA test for *Cxx*
FDV, Fiji disease fijivirus
FVF, fibrovascular fluid (also known as xylem fluid or xylem exudate)
NIb, viral replicase
OD, optical density (= Absorbance)
PCR, polymerase chain reaction
PCR-ELISA
RSD, ratoon stunting disease
RNA, ribonucleic acid
dsRNA, double-stranded RNA
ssRNA, single-stranded DNA
SCMV, Sugarcane mosaic potyvirus

2.0 ABSTRACT

This project developed diagnostic assays for sugarcane pathogens using novel DNA detection technologies associated with the polymerase chain reaction (PCR). The bacterium *Clavibacter xyli* subsp. *xyli* (*Cxx*) causing ratoon stunting disease (RSD) was used as major model to compare assay platforms based on PCR-ELISA (Boehringer) and TaqMan™ real time PCR (Applied Biosystems). TaqMan was more sensitive, robust, and subject to less interference than PCR-ELISA. Laboratory tests and field trials using cultured *Cxx* cells and xylem fluids from RSD-infected cane demonstrated that TaqMan could detect fewer than 10 bacterial cells reliably, and was >100x as sensitive as previous ELISA and phase contrast microscopy methods. Quantitative TaqMan assays appeared to be congruent with these existing methods. TaqMan assays were also developed for Fiji disease fijivirus (FDV) and sugarcane mosaic potyvirus (SCMV). BSES can readily access this advanced technology via the Real Time PCR Facility at the University of Queensland. Compared to alternative methods the TaqMan assays are inherently time-efficient, robust, highly sensitive, quantitative, and are especially well suited for rigorous quality control. These tests are recommended for ongoing evaluation for quarantine and other purposes by the Australian sugar industry. TaqMan is a robust generic technology and assays can readily be developed for further pathogens if the need arises.

3.0 SUMMARY

3.1 General Summary (non-technical)

The goal of this project was to develop improved methods for the routine detection of sugarcane pathogens using novel diagnostic technologies based on the polymerase chain reaction (PCR). Such methods would be particularly useful for pathogens that:

- are difficult to diagnose by visual inspection of planting material;
- are currently absent from Australia or likely to be present in imported germplasm;
- have the potential to reduce yield.

Initially, we developed PCR-ELISA (Boehringer) assays and TaqMan™ (Applied Biosystems) real time PCR assay systems, using the bacterium that causes ratoon stunting disease (*Clavibacter xyli* subsp. *xyli, Cxx*) as major model pathogen. These two advanced PCR-based technologies, which use a highly specific hybridisation probe for the automated and reliable detection of target DNA in a sample, were compared to each other and to the existing industry test, termed EB-EIA, which does not use PCR.

We found that the TaqMan assay for *Cxx* was quantitative and ~10x more sensitive than the PCR-ELISA, and ~ 100x more sensitive than the existing EB-EIA using 1/200th the amount of xylem fibrovascular fluid (FVF) per assay (overall increased sensitivity of 20,000x compared to EB-EIA). The TaqMan assay was more robust than the PCR-ELISA, and was so sensitive that it was capable of reliably detecting fewer than 10 bacterial cells or DNA template molecules per reaction (potentially a single cell or DNA template), and indeed will detect low levels of contamination if care is not taken to exclude this possibility. Field tests were used to validate the TaqMan assay for routine disease screening compared to the existing EB-EIA assay, but insufficient time was available to validate the TaqMan assay for early detection of *Cxx* in quarantine material.

TaqMan™ assays were also developed for Fiji disease fijivirus (FDV) and sugarcane mosaic potyvirus (SCMV), representing double-stranded RNA and single-stranded RNA viruses respectively. These assays were used to demonstrate FDV in the planthopper vector that tramsmits this virus and in asymptomatic leaves of infected sugarcane, and SCMV in asymptomatic leaf and pith tissue of infected sugarcane.

Given the robust sensitivity, high throughput capabilities, and quality assurance procedures inherent in TaqMan, we believe this technology is suitable for routine use by the sugar industry for PCR-based tests, e.g. when a definitive &/or quantitative result is required. Further validation is required for TaqMan tests to be used for primary screening of imported germplasm, e.g. to replace growth and observation of canes under quarantine conditions. Such validation can only be achieved through routine analysis of "real world" samples.

It is recommended that resources be provided to BSES to assist the ongoing implementation and validation of TaqMan or equivalent real time PCR technologies as a generic platform for pathogen detection for industry purposes requiring more efficient and reliable PCR-based diagnostic protocols, and for strategic response to emergencies.

It is sometimes suggested that a diagnostic assay procedure can be "too sensitive" if it is able to detect extremely low levels of a pathogen. **Such a view implies that it is better to use a less sensitive technology to avoid possible doubt caused by false positives or contamination**. To the contrary, the TaqMan assays described above are highly specific for the pathogen even at the detection limit, and a positive result can readily be distinguished from a high background "noise". Hence we would argue that it is better to know what is actually present in a sample, enabling an informed decision to be made when considering further action.

3.2 Summary of project development in relation to original research plan

Microbial pathogens are of concern to the sugar industry, particularly those that:

- are difficult to diagnose by visual inspection of planting material;
- are currently absent from Australia or likely to be present in imported germplasm;
- have the potential to reduce yield.

This project used novel diagnostic technologies based on the polymerase chain reaction (PCR) to develop improved methods for the routine detection, identification and quantitation of pathogens likely to compromise the safe exchange of sugarcane germplasm.

The bacterium *Clavibacter xyli* subsp. *xyli* (*Cxx*) causing ratoon stunting disease (RSD) was used as the major model for method development and assessment. Currently, losses are contained by an active program of disease control using field monitoring and clean seed cane underpinned by an existing robust (but relatively insensitive) evaporative-binding, enzyme-linked immunosorbent assay (EB-EIA).

The project initially assessed PCR-ELISA, which uses immunological methods to detect antigenically-labelled PCR products. PCR-ELISA protocols for *Cxx* and Fiji disease fijivirus (FDV) were developed. Analysis of *Cxx* in field material identified inhibition of PCR by some samples and cross-contamination as potential problems for the routine diagnostic use of PCR-ELISA.

In June 1997 we acquired equipment that enabled the development of "real time PCR" assays using the TaqMan™ system. This novel technology uses a fluorescent probe to detect DNA products as they are formed during successive reaction cycles of the PCR. A TaqMan assay developed for *Cxx* had the following attributes compared to other assay systems:

- The TaqMan for *Cxx* was ~10x more sensitive than the PCR-ELISA, and was 100x more sensitive than the existing EB-EIA using 1/200th the amount of xylem fibrovascular fluid (FVF) per assay (overall increased sensitivity of 20,000x compared to EB-EIA).
- The effective detection limit for the TaqMan assay was <10 bacterial cells, and the technology is capable of detecting a single cell reliably if present in an assay tube.
- Real time PCR amplification kinetic patterns clearly validated positive results at the limits of detection, an option not available with EB-EIA or PCR-ELISA.
- The high sensitivity of the TaqMan assay enabled the detection of low-level contamination of samples that would be missed by other detection systems. This prompted the development of more careful sample management procedures to prevent the occurrence of false positives arising by cross-contamination.

TaqMan assays were also developed for: Fiji disease fijivirus (FDV, a double-stranded RNA virus), and Sugarcane mosaic potyvirus (SCMV, a single stranded RNA virus), and assessed as follows:

- TaqMan demonstrated the presence of virus in the planthopper vector that transmits FDV.
- TaqMan detected FDV and SCMV in both symptomatic and asymptomatic leaf and pith tissue, and appears to be suitable for routine use requiring validated results (quarantine) or quantitative viral titres (evaluation of resistance in breeding germplasms).

Further research is required to address the following issues and opportunities:

- TaqMan analysis of *Cxx* in some quarantine and field samples showed discrepancies in bacterial titre in fibrovascular fluid compared to EB-EIA and phase contrast microscopy.

- Further research is needed to develop protocols for the routine and reliable TaqMan detection of *Cxx* in cruder preparations such as cane juice which contains inhibitors of PCR.
- TaqMan appears able to detect strains of FDV currently present in Australia, and further research is necessary to determine if the TaqMan FDV assay detects overseas strains.
- **It is recommended that resources be provided to BSES to assist the ongoing implementation and validation of the TaqMan assays developed to date.**

  This will require the comparison of TaqMan with alternative methods of disease diagnosis during routine disease management by BSES, in order to assess the reliability and benefits of TaqMan as a primary tool for disease diagnosis, and to refine sampling etc. strategies.

Given the sensitivity, high throughput capabilities, and quality assurance procedures inherent in TaqMan, we believe this technology is suitable for routine use by the sugar industry, subject to the validation process described above.

We are confident that any pathogen system suited to nucleic acid detection can readily be adapted to a TaqMan assay, and predict that real time nucleic acid assays will eventually replace end-point detection systems such as gel electrophoresis and PCR-ELISA for routine diagnostic purposes. TaqMan assays have also been developed for the fungal phytopathogens *Pachymetra chaunorhiza*, *Pythium arrhenomanes* and *P. graminicola*, and will be the subject of a separate SRDC report.

Issues of costs associated with this technology (IP licencing, reagents, equipment) need to be addressed and are discussed in detail in this report. Relatively high costs are not a problem for small numbers of high value samples requiring a definitive result such as breeding germplasm. However, we believe that similar costs will apply to alternative nucleic acid-based diagnostic technologies currently available and to be developed in the future, and that competition and the development of improved equipment will reduce costs in the longer term.

4.0 BACKGROUND TO THE PROJECT

4.1 Overall Aim:

The aim of this project as stated in the initial application was "**to develop improved, DNA-based methods for the routine detection, identification and quantitation of a range of sugarcane pathogens.**" Recent developments in DNA detection technologies associated with the polymerase reaction were to be assessed for use by the sugar industry.

4.2 The Project in relation to industry issues:

The project was targeted to SRDC R&D Program 3 (Crop Protection), particularly Strategy 3.4 "**Develop methodologies for safe exchange of germplasm through monitoring and diagnosis; production of disease-free planting material**". In this regard, diseases caused by microbial pathogens are of concern to the sugar industry for a number of reasons including:

- many pathogens present in Australia have a demonstrated potential to reduce yield,
- many exotic pathogens currently absent from Australia have the potential to reduce yield if introduced,
- the possibility that sugarcane germplasm may harbour latent or "hidden" pathogenic microbes restricts the transfer of breeding and planting materials to (and within) Australia'
- many sugarcane pathogens are difficult to detect by disease symptoms or microscopic observation.

Diagnostic assays developed for germplasm analysis require a high degree of reliability to avoid pathogen "escapes" or the needless destruction of valuable breeding stock, and assay methodologies should ideally discriminate positive results from false positives and false negatives. It is also of advantage to develop common assay platforms so the same generic technology can be applied to a range of different pathogens. We initially proposed to use the bacterium *Clavibacter xyli* subsp. *xyli* (*Cxx*) causing ratoon stunting disease (RSD), and the viruses Fiji disease fijivirus (FDV) and Sugarcane mosaic potyvirus (SCMV), as test pathogens to develop and validate such assays. Disease symptoms associated with these pathogens are difficult to diagnose in many situations.

Rigorous diagnostic technologies developed for germplasm analysis should also have application to disease management of indigenous pathogens, and indeed, routine testing of field-diseased material is desirable to validate the reliability of assays used to screen germplasm accessions. The test pathogens *Cxx*, FDV and SCMV are currently present in Australia where they are managed by hygiene, local quarantine or deployment of resistant varieties, and hence can be used to assess the generic efficacy and benefits of the improved diagnostic technologies developed in this project.

Clavibacter xyli subsp. *xyli* (*Cxx*) causing ratoon stunting disease (RSD) was used as the major model for method development and assessment. RSD represents a continuing industry problem estimated to cause a $30m loss annually to the Australian sugar industry. Losses are contained by an active program of disease control using field monitoring and clean seed cane underpinned by an existing robust (but relatively insensitive) evaporative-binding enzyme-linked immunosorbent assay (EB-EIA) for *Cxx* detection.

4.3 Technical Considerations:

Nucleic acid (DNA and RNA) sequences are available for many of the pathogens of interest to the Australian sugar industry. This project proposed to use pathogen-specific "signature sequences" to design oligonucleotide DNA primers that specifically amplify such sequences via the polymerase chain reaction (PCR). Specific DNA hybridisation probes designed from internal regions of an amplified signature sequence make it possible to develop simplified methods for automating the detection and quantitation of a particular pathogen, and also provide a second level of specificity to validate a positive result. **It was initially proposed that two generic assay systems using internal hybridisation probes, termed PCR-ELISA and TaqMan, be developed and assessed**. Such tests would be able to detect specific sugarcane pathogens in the presence of DNA from other microorganisms, host tissue, or soil.

Research sponsored by the CRC for Tropical Plant Pathology had substantially developed a PCR-ELISA assay for *Cxx* prior to the start of Project UQ024 in January 1997. PCR-ELISA was chosen as first system to develop automated assays as it uses PCR to amplify antigenically-tagged DNA copies of a target sequence, and the tagged copies are detected via a hybridisation capture probe by modifications of standard ELISA technology as used for the existing EB-EIA assay for *Cxx*. It was intended to validate this PCR-ELISA test in Brisbane and transfer the technology to regional laboratories for routine use.

We also proposed to develop TaqMan "real time PCR" assays if submissions to the Australian Research Council for the purchase of enabling equipment were successful. TaqMan™ (developed by Applied Biosystems) uses a hybridisation probe that releases a fluorescent reporter dye only when a specific PCR product is amplified. The fluorescence is detected in "real time" during the progress of PCR using a thermal cycler capable of making fluorescence measurements in reaction tubes during successive steps of the chain reaction. This real time capability simplifies the effort required to obtain a result by eliminating all post-PCR manipulations other than computer analysis, and real time technologies that simultaneously amplify and detect signals derived from specific nucleic acid templates are expected to represent the DNA-based diagnostics of the future.

Further details of the PCR-ELISA and TaqMan technologies are presented in Section 6.0

5.0 OBJECTIVES & STATEMENT OF ACHIEVEMENT

5.1 Full Project Objectives as stated in Initial Project Application

This project proposes to develop improved diagnostic procedures for the routine, efficient and reliable identification and analysis of a variety of sugarcane pathogens, using the most recent advances in DNA technology based on the polymerase chain reaction (PCR). This will be achieved by:

1. Identification of "signature" nucleotide sequences unique to the genomes of targeted microbial pathogens of sugarcane, using information from previous or cognate research.
2. Selection or design of oligonucleotide PCR primers and internal hybridisation probes specific to species and strains of sugarcane pathogens of concern to the sugar industry. These primers and probes will be tested and redesigned as necessary to achieve adequate specificity and sensitivity.
3. The above primers and probes will be used to develop advanced PCR-based formats for the sensitive detection and identification of sugarcane pathogens in field samples. Initially, we will develop "PCR-ELISA" formats that adapt standard serological equipment to DNA detection. For viruses we will also develop "immunocapture PCR" for increased sensitivity. "TaqMan", an innovative new format that simplifies detection and facilitates quantitation, will be developed when appropriate.
4. By interaction with the users of diagnostics tests in the sugar industry (eg. BSES regional and quarantine laboratories), these formats will be adapted to routine screening and research applications.
5. Priority will be given to completion of the current CRCTPP/QABC/BSES project on the development and implementation of PCR-ELISA for diagnosis of ratoon stunting disease (RSD) caused by the bacterium *Clavibacter xyli* subsp. *xyli*. Subsequent priorities will be viral diseases, initially sugarcane mosaic potyvirus (SCMV) and Fiji disease reovirus (FDV), followed by known strains of sugarcane bacilliform badnavirus (SCBV), yellow leaf syndrome (YLS) and chlorotic streak when characterised, and other pathogens (eg. the fungus *Pachymetra chaunorhiza*) according to industry priorities at the time.

5.2 Statement of Achievement of Project Objectives:

Dr. Juliane Henderson was appointed in January 1997 (initially as Research Assistant, moving to Research Officer when her PhD was finalised in 1998). The objectives stated above were achieved as follows:

1. Suitable signature sequences were found for the prioritised pathogens *Cxx*, FDV and SCMV.
   (Achievement of Objectives 1).

2. Sensitive and specific PCR primers and internal hybridisation probes specific to *Cxx*, FDV and SCMV were designed and validated for specificity and sensitivity.
   (Achievement of Objective 2).

3. PCR-ELISA assays were established for *Cxx* and FDV, and TaqMan assays were developed for *Cxx*, FDV and SCMV. The sensitivity of the TaqMan assays developed for FDV and SCMV made it unnecessary to develop immunocapture PCR for reliable virus detection.

(Achievement of Objectives 3 and 5)

4. The PCR-ELISA and TaqMan assays noted above were assessed using laboratory, glasshouse and field material obtained in collaboration with BSES. Specific achievements are listed below in Section 5.3.
(Achievement of Objective 4).

5.3 Summary of key issues and problems addressed during the project:

(i) The PCR-ELISA for *Cxx* was set up and trialled at the regional BSES Research Station at Tully in the first half of 1997. Assays of xylem fibrovascular fluids (FVF) from field canes at Tully and our laboratories at UQ St. Lucia identified cross-contamination (probably the products of previous assays), and inhibition of the PCR reaction using FVF collected from some sugarcane varieties, as potential problems for the routine use of PCR-ELISA.

(ii) D.J. Maclean received Australian Research Council (ARC) and other funding for an ABI-PRISM Model 7700 Sequence Detector (purchased June 1997), enabling the development of TaqMan™ PCR (or reverse-transcriptase PCR [RT-PCR]) assays for *Cxx*, FDV and SCMV. The TaqMan assay for *Cxx* was more robust, ~10x more sensitive, and less prone to interference than the PCR-ELISA assay, which in turn was ~10x more sensitive than the existing EB-EIA assay. Compounding this increased sensitivity, the PCR-ELISA and TaqMan assays each used 1 μL of sample per assay compared to 200 μL for the EB-EIA.

(iii) Highly sensitive single-tube RT-PCR-TaqMan assays were developed for FDV and SCMV. The TaqMan assays for *Cxx*, FDV and SCMV were quantitative over about six orders of magnitude of purified nucleic acid templates, with detection limits of <10 nucleic acid templates (the effective detection limit is dictated by the homogeniety of template in sample fluids, and a single template can be detected if present in a reaction tube). This increased sensitivity of PCR product detection with TaqMan made it unnecessary to develop immunocapture PCR for reliable virus detection.

(iv) Because the TaqMan assays for *Cxx* and FDV were more robust and sensitive than PCR-ELISA, were less prone to interference and cross-contamination, and were quantitative over a wider dynamic range, we concluded that TaqMan was the more robust technology and ceased further development of PCR-ELISA.

Subsequent assays of quarantine material and field samples produced the following findings:

- Real time TaqMan amplification patterns enabled validation of positive or negative results at the detection limits of the assay system. This validation was especially useful for field samples with low *Cxx* titres. Assay systems that rely on end-point detection, such as EB-EIA or PCR-ELISA, are difficult to validate at their detection limits.
- The extreme sensitivity of TaqMan prompted the development of more careful sample management procedures to avoid cross-contamination (cf. Section 5.3 below).
- TaqMan analysis of some quarantine and field samples showed discrepancies in bacterial titre compared to alternative test methodologies (ELISA and phase contrast microscopy). Further research is necessary to resolve these disparities.

- Further research is also needed to develop protocols for the routine and reliable detection of *Cxx* in cruder preparations such as cane juice and pith samples.
- The FDV TaqMan assay appears to be capable of detecting virus strains currently present in Australia. Checks need to be made on overseas material known to be infected with FDV.
- The FDV PCR-ELISA demonstrated the presence of virus in the planthopper vector known to transmit disease.
- The FDV TaqMan assay demonstrated that virus was present in planthopper nymphs emerging from FDV-infected cane, suggesting that FDV can be transmitted from adult planthoppers to their progeny via the reproductive system.
- TaqMan assays for FDV and SCMV detected virus in both symptomatic and asymptomatic leaf and pith tissue, and appear to be suitable for routine uses such as those requiring validated results (quarantine) or quantitative viral titres (evaluation of resistance in breeding germplasms).

5.4 Project Reviews:

Two project reviews were held (Review I: September 1998, and Review II: September 1999) to consider industry priorities in relation to results, in particular (i) the replacement of PCR-ELISA with TaqMan technology, (ii) the consequences of the increased sensitivity of TaqMan, which appeared to generate false positives from canes believed to be RSD-free in a key field trial, and (iii) to prioritise further research. The following issues emerged as a consequence of the reviews:

Review I. Revision of the project to focus on the development and assessment of TaqMan technology was endorsed, and it was noted that the research had been conducted with scientific and technical rigour. The apparent false positives found in supposedly RSD-free cane were a matter of concern to be addressed at a later review.

Review II. The second review considered further research that addressed the problem of apparent false positives in putatively RSD-free cane. A major conceptual problem with novel ultra-sensitive analytical techniques is that low-level contamination unseen by previous established methodologies can now be detected.

Results of TaqMan assays were presented that showed the absence of *Cxx* in putatively RSD-free field cane samples when improved sample containers were used to collect and transport xylem fibrovascular fluids (FVF). Data were presented showing that TaqMan results at the limits of detection could readily be classified as true or false by inspection of the real time spectral kinetics accumulated during each PCR run ("multicomponent analysis"). Experience suggested that the inherent specificity of TaqMan is such that a positive amplification signal cannot be given by amplification of an incorrect target sequence, and that amplification of even a single target template gives unambiguous exponential kinetics. Routine procedures for clean PCR reaction set-up to avoid cross-contamination are essential. Because TaqMan assays are run and analysed in closed reaction tubes which can then be discarded, the potential to cause cross-contamination can be better minimised by careful hygiene than alternative PCR detection technologies requiring electrophoresis, blotting or staining etc. of the PCR products.

It is sometimes suggested that a diagnostic assay procedure can be "too sensitive" if it is able to detect low levels of contamination. Such a view implies that it is better to use a less sensitive technology to avoid overt doubt. **To the contrary, we would argue that provided the assay procedure is highly specific for the pathogen, and that a high background "noise" cannot be confused with a positive result, it is better to know what is actually present in a sample, enabling an informed decision to be made when considering further action.** The detection of even a single pathogen template might indicate the presence of other templates in the tissue of origin and could represent (for example) low titres from an early stage of infection. One of the virtues of real time TaqMan-PCR assays is that a positive result at the limit of detection is easily recognised by the kinetics of specific DNA amplification, and can be readily distinguished from background fluorescence signals. TaqMan also makes it possible to re-test suspect cane samples at high sensitivity with a high degree of quality assurance.

(iii) Revision of Milestones. Milestones were revised to prioritise a detailed assessment of the TaqMan assay system for *Cxx*, resulting in insufficient time being available to develop TaqMan assays for further pathogens other than FDV and SCMV (assays have also been developed for *Pachymetra* and *Pythium* as part of the PhD project of L. Heelan, and will be the subject of a separate SRDC Report). Milestones were also revised to include the development of an endogenous internal control to allow the development of multiplexed assays to identify false negatives; however, insufficient time was available to achieve this aim due to ongoing assessment of the *Cxx* assay.

This report has been prepared in collaboration with Mr. Barry Croft of BSES, and it is intended that the TaqMan assays will be checked against alternative methods of pathogen detection (e.g. long-term growth to allow disease symptoms to develop) in ongoing tests of quarantine and field material. This longer term perspective will assist BSES to evaluate the benefits and limitations of nucleic acid-based diagnostics tests in general, and to define the circumstances when such tests (including TaqMan as a highly sensitive and quantitative system for conducting nucleic acid tests) should be used for pathogen detection.

6.0 METHODOLOGY AND ITS JUSTIFICATION

6.1 Choice of pathogen systems for development of diagnostic assays

The technical strategy underlying this project was to detect pathogens by amplifying signature sequences in their genomes via the polymerase chain reaction (PCR). Pathogen-specific sequences can enable PCR to detect very small amounts of a pathogen in host or environmental samples (e.g. leaves, pith, roots or soil) without first having to obtain pure cultures of the pathogen. For most pathogens pure culturing is time-consuming, and in many situations is difficult or impracticable. The three pathogens chosen to develop methodologies for this project illustrate these principles. Our ultimate intention was to develop a generic diagnostic platform where a variety of different sugarcane pathogens could be detected by sensitive, robust, reliable and time-efficient PCR-based tests under uniform assay conditions. Ability to quantify pathogen titres accurately was seen as a highly desirable bonus and was assessed routinely during method work-up and assessment.

***Clavibacter xyli* subsp. *xyli* (*Cxx*)** grows in the xylem of sugarcane, and in pure culture is very slow-growing and nutritionally fastidious. Thermal cycling during the denaturation step of PCR can release the DNA genome from intact bacterial cells. This can enable DNA extraction methodologies to be avoided if bacterial suspensions are supplied in a matrix that does not interfere with the enzyme-catalysed steps of PCR. Because other pathogens and nutritional/environmental conditions can cause the disease symptoms frequently associated with RSD (growth stunting and reddening of vascular tissue in stalk nodes), it is necessary to demonstrrate the presence of the pathogen for positive diagnosis of RSD. Existing tests include phase contrast microscopy and an ELISA, and are relatively insensitive with a lower detection limit of ~10^6 bacterial cells/mL (10^3 cells/μL); neither of these tests are totally specific for *Cxx*. The disease is controlled by hygiene and replanting, and 30-50,000 ELISA tests are conducted per year to identify *Cxx*-infested crops and to confirm the RSD-free status of clean seed cane. This existing test provides an ideal "benchmark" to evaluate the generic benefits and problems associated with the newer PCR-based tests which tend to be more sensitive and specific than the previous tests.

Fiji Disease Fijivirus (FDV) has a double-stranded RNA genome and forms definitive whitish galls associated with vascular bundles on the undersurface of leaf laminae. Further symptoms appear as the disease progresses and stalk development slows, successive leaves become shorter, harsher and stiffer, the stalk apex develops a fan-like appearance and in severe cases the leaves appear to be "bitten off". If infected with FDV, plants may exhibit varying degrees of stunting when they develop from diseased sets or ratooned root stocks (Egan et al., 1989). Disease symptoms may develop within two weeks or take as long as several months, depending on the cane variety, growth rate of the plant, and age of the plant at infection. Older cane, infected late in the growing season, may not express symptoms for many months, and sometimes only after ratooning (Egan et al., 1989), and sensitive assays capable of detecting virus in asymptomatic tissue would be useful. The disease is spread by delaphacid leafhoppers in the genus *Perkinsiella*, of which only *P. saccharicida* is known in Australia. Reliable methods of detecting virus in individual hoppers or nymphs from newly-hatched eggs would have applications in disease management and research.

Sugarcane mosaic potyvirus (SCMV) is an important pathogen of sugarcane in Australia, causing significant yield losses in susceptible cane varieties (Teakle and Grylls, 1973). A member of the economically important potyvirus group, SCMV has a single-stranded RNA genome which has been shown to exhibit considerable nucleotide sequence variability (Handley *et al.,* 1996, Handley *et al.,* 1998). In sugarcane, SCMV causes a systemic mosaic and reduction

in crop yield. The virus is transmitted by insect vectors in the field but also is transmissible mechanically, by grafting and in seed. Diagnosis of SCMV can be carried out via host range testing on sorghum, maize and many dicotyledonous plants, however, these methods are time-consuming and not always definitive. While there are many strains of SCMV in the SCMV/sorghum mosaic potyvirus complex, only one strain (SCMV-A) is currently in Australia. A reliable method capable of detecting all Australian field isolates of SCMV-A would assist greatly in control of the virus.

6.2 Selection of signature sequences, sources of cloned control templates, etc.

Clavibacter xyli subsp. *xyli* (*Cxx*). In prior research, Fegan et al (1998) sequenced the internal transcribed spacer (ITS) region of ribosomal RNA genes encoding the small and large subunits (SSU & LSU) of ribosomal RNA for *Cxx* and a related bacterium, *C. xyli* subsp. *cynodontis* (*Cxc*). *Cxc* has been isolated from the xylem of various other grasses, including Rhodes grass which is often grown adjacent to canelands. Significant differences in the ITS enabled the design of gel-based, species-specific PCR assays which showed that *Cxc* appeared to be absent from RSD cane infected with *Cxx*, and *Cxx* appeared to be absent from *Cxc*-infected Rhodes grass (Fegan et al, 1998). Although these and other data show that *Cxc* appears to be a non-pathogen of sugarcane, we nonetheless designed PCR primers and internal oligonucleotide primers for PCR-ELISA and TaqMan assays that were specific for *Cxx* compared to *Cxc* (Figure 6.1). A clone named *p*Clavi, that contains the complete ITS region of *Cxx* was supplied by Dr Mark Fegan (UQ, Microbiology) and was used as a positive control template.

Fiji Disease Fijivirus (FDV). FDV has a dsRNA genome consisting of 10 linear fragments ranging in size from 1.7 to 4.4 kb, with a total size of ~30 kb (Smith & Van de Velde, 1994). FDV is packaged into icosahedral particles ~70 nm in diameter with an unstable outer coat with 12 protrusions at their vertices. Smith et al (1992) sequenced portion of one of the 1.7 kb genome fragments and developed a gel-based RT-PCR assay that ampified a 450 bp DNA product which could detect 100 ag of purified FDV dsRNA. Figure 6.2 shows the position of oligonuleotide primers and hybridisation probes for PCR-ELISA and TaqMan assays within this 450 bp amplified sequence. The 450 bp amplified DNA sequence was cloned into the plasmid vector pGEM-T Easy; one of these clones, named pPFG2-6 (derived from RT-PCR-amplified FDV collected from infected sugarcane at the BSES Pathology Farm at Eight Mile Plains) is available for use as a positive control.

Sugarcane mosaic potyvirus (SCMV) Considerable research has been carried out investigating the sequence diversity of Australian isolates of SCMV (Handley *et al.,* 1996, Handley *et al.,* 1998). In these studies, sequences from the replicase (NIb) coding region of eight Australian isolates and the coat protein (CP) coding region of twelve isolates were examined. Using these data, TaqMan assays were designed to conserved regions within each of the NIb (Figure 6.3) and CP coding regions (Figure 6.4). Initial experiments proved the CP coding region assay to be the more robust of the two and subsequent experiments focused on this system. A plasmid containing the coat protein sequence of a common Australian SCMV strain under the control of the bacterial T7 RNA promoter was constructed for use in the generation of RNA transcripts. Single-stranded RNA copies homologous to the SCMV CP were produced *in vitro* using T7 RNA polymerase and these transcripts were used as a positive control and for the preparation of an SCMV dilution series for quantification.

6.3 Collection, storage and management of biological samples

Clavibacter xyli subsp. *xyli (Cxx)* The Australian sugar industry routinely employs EB-EIA to screen cane fibrovascular fluid (FVF), also known as xylem fluid or xylem exudate, for RSD. Collection of FVF is relatively simple and FVF contains few cellular substances likely to cause interference in assays. Therefore, it was decided that this sample type would also be used in PCR-ELISA and TaqMan assays for RSD. To minimize the risk of cross-contamination, our final sampling procedures collected FVF into screw-capped, O-ring, 2mL Eppendorf tubes. The use of screw-capped lids prevents leakage and aerosols which commonly occur when flip-top lids (eg. standard Eppendorf tubes) are used. Samples were transported on wet ice and stored at –20°C. Details of the FVF collection procedure can be found in Appendix 2.

In addition to FVF, pith tissue was investigated as a possible sample type. Internodal pith tissue was collected from the bottom of the cane stalk by firstly splitting the stalk lengthways using sterile secateurs and then excising a piece of tissue approximately 5mm x 20mm using a clean scalpel blade. The excised tissue was placed in a 2mL screw-capped, O-ring collection tube and 500µL of sterile water was added. The samples were left at 4°C overnight to allow any *Cxx* present in the pith tissue to exude into the water. Aliquots of the water were then analysed directly via EB-EIA or TaqMan.

Fiji disease fijivirus (FDV) Where possible, gall tissue was taken for analysis. In the absence of visible galls, tissue from the leaf mid-rib and/or leaf laminae was taken instead. Leaf tissue samples were stored at –80°C until required for use. Planthoppers (*Perkinsiella saccharicida*) were collected using a leaf blower device on reverse-cycle mode. Planthoppers were stored either in Eppendorf tubes at –80°C or under ethanol at 4°C. Planthopper nymphs were raised from eggs deposited by adult planthoppers in infected leaf tissue. The oviposition sites were excised and incubated at 28°C for 14 days in a petri dish lined with moist filter paper. Emerging nymphs were collected and stored at –20°C until required.

Sugarcane mosaic potyvirus (SCMV) Leaf tissue, symptomatic and asymptomatic, was collected and stored fresh at –80°C. Desiccated quarantine leaf samples were also stored at –80°C upon receipt.

6.4 Nucleic acid extraction and purification etc.

Extraction of total nucleic acid from sugarcane tissue (FDV, SCMV) and planthoppers (FDV)

Total Nucleic acid extraction of sugarcane tissue (pith and leaf tissue) Two methods for total nucleic acid extraction from sugarcane leaf tissue were used (See Appendix 3). The first, a rapid release protocol (Thomson and Dietzgen, 1995), involved boiling the leaf tissue in an extraction buffer to release nucleic acids and used the extract directly in PCR. This method produced a very crude extract as it included no clean-up steps. The second was the FastRNA™ Kit – Green (Bio101). This kit incorporates ceramic beads to disrupt cell tissues when shaken at high speeds and phenol chloroform extraction to produce a very clean end product. The combination of these factors allows a rapid procedure, decreasing labour-time while decreasing the time in which RNA could potentially be degraded.

Total nucleic acid extraction for FDV detection in Planthoppers Total nucleic acid was extracted from *Perkinsiella saccharicida* insect vectors using a combination of high pH and phenol as described by Robertson *et al.,* 1991 (See Appendix 3). Preparations were stored at –80°C in sterile Eppendorf tubes.

Preparation of sugarcane pathogen controls

Purfication of FDV dsRNA from infected sugarcane Bulk extraction of dsRNA from gall tissue of FDV-infected sugarcane was carried out as per the method of Dale *et al.,* (1986) (See Appendix 3). Following extraction, the RNA was analysed for quality on a non-denaturing 10% polyacrylamide gel stained using silver nitrate (Bassam *et al.,* 1991). The RNA was then quantified by spectrophotometry using a GeneQuant RNA/DNA calculator and stored aliquotted at –80°C.

In vitro transcription of SCMV Coat Protein RNA RNA transcripts of the SCMV coat protein (CP) coding region were generated from a plasmid clone constructed from pCR-SCRIPT (Stratagene) containing the CP and 3' untranslated region sequences of an Australian isolate of SCMV. Specific procedures leading to the production of RNA transcripts can be found in Appendix 3**.**

6.5 Principles of the polymerase chain reaction (PCR) and definition of terms

The polymerase chain reaction (Saiki *et al.,* 1986) uses two oligonucleotide primers homologous to each end of a specific sequence in a double-stranded DNA template. Recombinant or purified DNA polymerase derived from a thermophile e.g. the bacterium *Thermus aquaticus* (source of "*Taq* polymerase") in the presence of deoxynucleoside triphosphates (dNTPs) and a suitable buffer, amplifies the sequence exponentially via successive 3-step reaction cycles. In the first ***denaturation*** step, the reaction mixture is heated to ~94°C to denature the dsDNA to ssDNA strands. In the second ***annealing*** step, the mixture is cooled to a specified ***annealing temperature*** (e.g. 60°C) to allow the oligonucleotide primers to hybridise to the denatured template strands. As the primers bind to the template, DNA polymerase starts to extend the 3'-end of each primer. A third ***extension*** step raises the temperature to the optimum for DNA polymerase activity (72°C for Taq polymerase). However, DNA polymerase is active (albeit more slowly) at the annealing temperature, and for short DNA sequences the extension step can be omitted and the extension process can be completed during ***ramping*** from annealing to denaturation (60→95°C) to start the next amplification cycle. This two-step protocol is used for TaqMan assays.

Because DNA polymerase is active at lower temperatures than the optimum 72°C, non-specific priming can occur during reaction set-up at room temperature, and can lead to the production of false positives during diagnostic PCR. This is most easily avoided by hot start PCR, e.g. by using a recombinant AmpliTaq Gold (Applied Biosystems) that is only activated by heating at 95°C for 5-10 minutes prior to starting the thermal cycling. False positives are also avoided by detecting the PCR product using a specific hybridisation probe homologous to an internal sequence within the product as described for PCR-ELISA or TaqMan (below).

Single stranded RNA (e.g. SCMV) can be unstable in tissue extracts and requires more careful sample management than double-stranded genomic DNA from cellular pathogens such as bacteria (e.g. *Cxx*) or fungi. A reverse transcriptase step is necessary to make a complementary DNA copy (cDNA) of the signature sequence in the SCMV genome prior to PCR. The combination of reverse transcriptase followed by PCR is termed RT-PCR. Double-stranded

RNA (e.g. FDV) is more stable than ssRNA and can be purified more easily from plant extracts, but needs to be heated with oligonucleotide primers prior to the reverse transcriptase step, an extra manipulation that complicates the adaptation of many RT-PCR protocols to dsRNA templates. These two viruses (SCMV, FDV) and *Cxx* provided examples of the different types of nucleic acid templates likely to be assayed using generic PCR protocols developed for a variety of sugarcane pathogens.

6.6 PCR-ELISA assays

The PCR-ELISA kit (Roche) was adapted to detect both *Cxx* and FDV.

The PCR-ELISA assays for *Cxx* and FDV use specific primers in a modified PCR which allows the incorporation of an antigen (digoxygenin) into the DNA during the reaction. Reaction products are then hybridised, in solution, to a biotinylated DNA probe specific to an internal region of the amplified sequence. The hybridisation products are immobilised onto the surface of a microtitre plate well coated with streptavidin (which has a specific affinity for biotin) and non-specific materials are removed in a series of washing steps. The bound hybrid is detected by an anti-digoxygenin peroxidase conjugate and by use of the colorimetric substrate ABTS®. Specific details of the PCR-ELISA technique and assay protocols are given in Appendix 4 of this report.

The PCR-ELISA test for RSD is based on signature DNA sequences in the intergenic spacer (ITS) region of genes encoding ribosomal RNA in *Clavibacter xyli* subsp. *xyli (Cxx)* (Fegan *et al.,* 1998). Primers *Cxx*ITSF#5 and *Cxx*ITSR#5 were used to amplify a 304bp region of the *Cxx* ITS (Figure 6.1). The PCR-ELISA test for FDV is designed to one of the 1.7kb dsRNA linear segments of the FDV genome using primer designed previously for use in an RT-PCR method (Smith *et al.,* 1994, Pickering, 1997). Primers FDV 7F and FDV 7R were used to amplify a 444bp region of the segment which was then detected using the internal hybridisation probe FDV-Bio-7 (Figure 6.2).

6.7 TaqMan assays

The TaqMan system utilises the 5'-nuclease assay which incorporates a sequence-specific internal probe to enable reproducible quantitative PCR results (Figure 6.5). The probe is labelled with a fluorescent dye at the 5'-end and a quencher dye at the 3'-end. During PCR, the probe hybridises between the sequence specific forward and reverse primers. When this probe is intact, very low levels of fluorescence are emitted by the reporter dye, as the radiant energy captured by the reporter is transmitted to the quencher dye via a mechanism known as Fluorescence Resonance Energy Transfer (FRET). As extension proceeds, the 5'-nuclease activity of *Taq* DNA polymerase cleaves the probe, separating the reporter dye from the quencher dye and allowing an increase in reporter dye fluorescence. The ABI Prism™ 7700 Sequence Detection System enables "real-time" measurement of this increase in fluorescence which is quantitatively related to the amount of starting template.

TaqMan assays to detect RSD, FDV and SCMV in sugarcane were designed using Primer Express 1.0 software (Applied Biosystems). This software has been specially developed to assist the design of TaqMan but can be used for other PCR applications. We have found it to be very reliable.

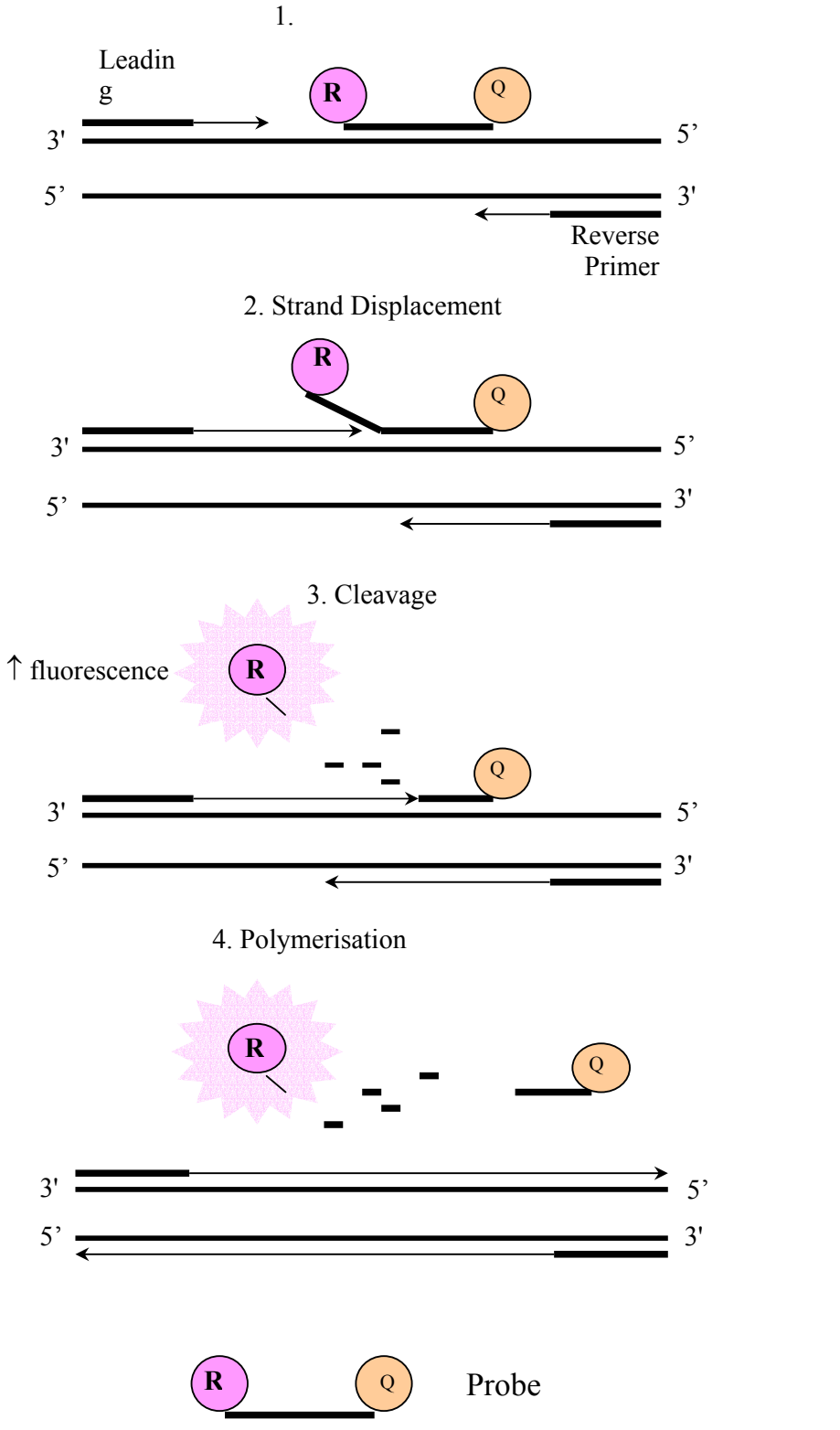

Figure 6.5 Diagrammatic representation of the TaqMan 5'nuclease activity.

Clavibacter xyli* subsp. *xyli (Cxx) The TaqMan assay for RSD was designed to the 16S-23S rRNA ITS region of *Cxx* using sequence of an Australian isolate (Fegan *et al.,* 1998) (Figure 6.1). Specific PCR primers *Cxx*TaqF1 and *Cxx*TaqR1 amplified a 68bp fragment of the ITS region and the *Cxx* specific probe CLAVTAQ1 hybridised to an internal region of this fragment. TaqMan assay reactions were set up using either the TaqMan PCR Core Reagents Kit (Applied Biosystems) or the TaqMan Universal PCR Master Mix (Applied Biosystems). Full details of the PCR setup, reaction components and thermal cycling protocols are located in Appendix 5.

Fiji disease Fijivirus (FDV) The TaqMan assay for FDV was designed to one of the 1.7kb dsRNA linear segments of the FDV genome (Smith *et al.,* 1994). Specific primers FDV727F and FDVTq-R amplified a 236bp product of this segment and the specific probe FDV-FAM-7 hybridised internal to these primers to give a second level of specificity (Figure 6.2). TaqMan assay reactions were set up using the TaqMan EZ RT-PCR Core Reagents Kit (Applied Biosystems). This assay replaces *Taq* DNA polymerase with *Tth* DNA polymerase, a thermostable enzyme that has both reverse transcription (RT) and DNA polymerase activity. This allows the two steps of RT-PCR to be done in the same tube for a dsRNA template. Full details of the PCR setup, reaction components and thermal cycling protocols are located in Appendix 5**.**

Sugarcane mosaic potyvirus (SCMV) Two TaqMan assays, based on different coding regions, were designed to detect SCMV. The coat protein (CP) and replicase (NIb) coding regions were selected due to the high conservation of these sequences. Use of highly conserved sequences decreases the chance of failing to detect isolates due to variability in sequence. Sequence specific primers SCMVREPf and SCMVREPr amplified a 78bp fragment of the NIb coding region which was subsequently detected using the internal hybridisation probe SCMVTaqREP (Figure 6.3). For the CP coding region, specific primers SCMVCPf and SCMVCPr amplified a 63bp fragment which was detected using the hybridisation probe SCMVTaqCP (Figure 6.4). TaqMan assay reactions were set up using the TaqMan Gold RT-PCR Core Reagents Kit (Applied Biosystems). Full details of the PCR setup, reaction components and thermal cycling protocols are located in Appendix 5**.**

7.0 DETAILED RESULTS AND DISCUSSION

7.1 Development of a PCR-ELISA for Cxx

A PCR-ELISA assay was developed to detect *Cxx* cells in sugarcane fibrovascular fluid (FVF). The assay targeted the 16S-23S rRNA ITS region of *Cxx* and is capable of discriminating between *Cxx* and the closely related pathogen, *Clavibacter xyli* subsp. *cynodontis* (*Cxc*). A 304bp fragment was amplified using primers *Cxx*ITSF#5 and *Cxx*ITSR#5 and a second level of specificity was conferred using a biotin-labelled internal hybridisation probe specific to this sequence.

The PCR-ELISA assay for *Cxx* was sensitive and semi-quantitative to a level of 50-100 cultured cells/per 1µL sample (Figure 7.1). This value (~10^5 cells/mL) is approximately 10 times more sensitive than EB-EIA (detection limit of ~10^6 cells/mL). In addition, the sample volume for PCR-ELISA was 1µL compared to 200µL for EB-EIA which improved sensitivity even further.

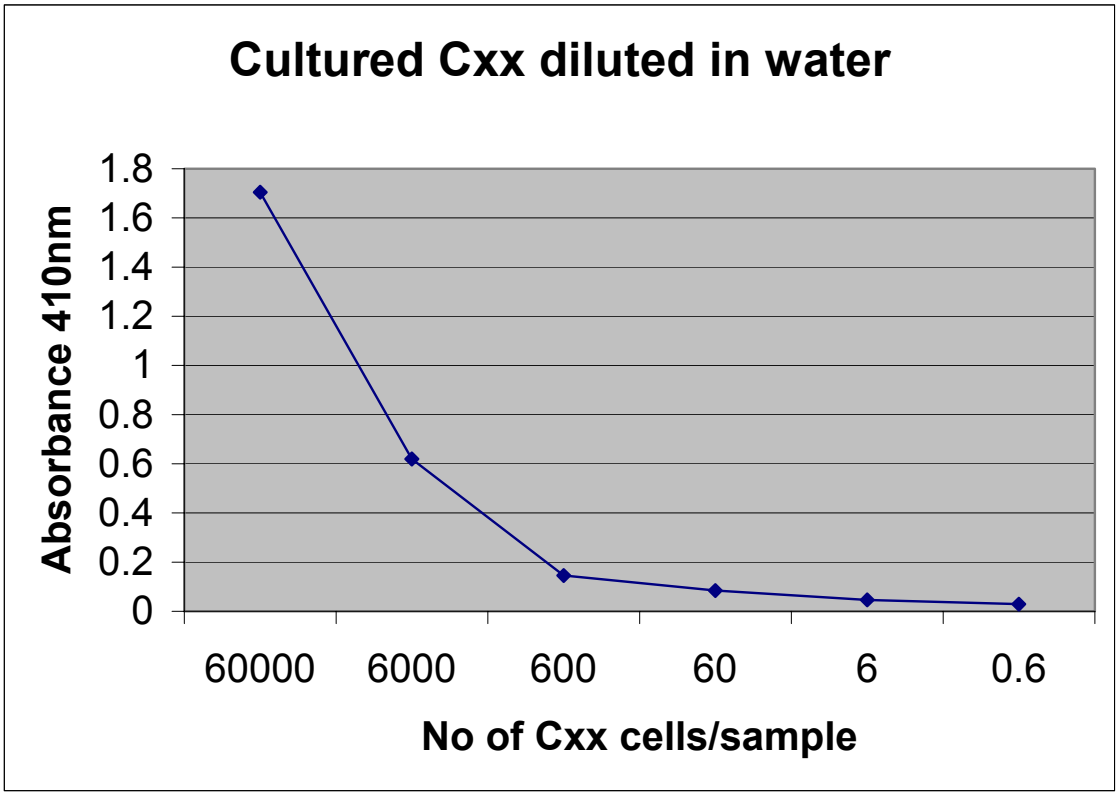

Figure 7.1 Serial dilution series of purified *Cxx* cells in water as assayed by PCR-ELISA showing absorbance values in microtitre wells after the ABTS component of the assay. The assay is sensitive and semiquantitative to a level of 50-100 cells/µL. (*Cxx* cells were kindly supplied by Lars Petrasovits, BSES, Indooroopilly)

This assay was transferred to the Bureau of Sugar Experiment Stations (Tully) in April, 1997. A manual was prepared describing the PCR-ELISA technique (See Appendix 4) and Dr Juliane Henderson spent a week at the Tully Research Station training laboratory staff in its use. Following this technology transfer, testing of the PCR-ELISA continued using field isolates of different cane varieties to test the robustness of the technique. During this assessment, difficulties in detecting *Cxx* in FVF sampled from a wide range of cane varieties were identified. Figure 7.2 demonstrates the variable results for detecting *Cxx* in differing cane varieties that were known to be infected with RSD. It was postulated that this variation resulted either from the presence of *Taq* inhibitors in the sample or from differing levels of *Cxx* titre conferred by the resistance phenotype of the cane variety.

7.2 Development of a TaqMan assay for the detection of Cxx

7.2.1 Sensitivity of the TaqMan assay

During further field testing of the *Cxx* PCR-ELISA, development of a real-time, TaqMan assay to detect *Cxx* was started. Initial comparisons with PCR-ELISA and EB-EIA, quickly highlighted the many advantages of real-time PCR technology.

Similarly to the PCR-ELISA assay for *Cxx* , a TaqMan assay was designed to the 16S-23S rRNA ITS region of *Cxx* and is capable of distinguishing *Cxx* from the closely related pathogen *Cxc* (the TaqMan assay reverse primer *Cxx*TaqF1 was designed to bind over a region of the *Cxx* genome that contains a large deletion in *Cxc*).

The assay was quantitative over six orders of magnitude of a $1x10^6$ to $1x10^o$ dilution series of the plasmid, pCLAVI and could detect a single plasmid template if present in a PCR reaction tube (Figure 7.3). Using purified *Cxx* cells to create a dilution series, the TaqMan assay was quantitative over the range ~10 to 10,000 bacterial cells/PCR reaction tube and was capable of detecting 10 cells per reaction (Figure 7.4) or less (data not shown).

Comparison of standard curves for plasmid and purified *Cxx* over numerous experiments showed the slope of the plots to be very similar (data not shown). Therefore, it was concluded that plasmid template could be used to generate standard curves for quantitation of field isolates. This is advantageous as preparation and storage of plasmid templates is far more convenient than using cultured *Cxx* cells.

7.2.2 Field Trial 1: Comparison of TaqMan & other methods for RSD demonstration plots

A pilot study was conducted to compare the relative efficiencies of EB-EIA, PCR-ELISA and TaqMan on samples taken from RSD-free and RSD-infected demonstration plots grown at the BSES Tully research station. A total of 16 putatively RSD-free canes and 16 putative RSD-infected canes were analysed and the comparison revealed increased sensitivity of PCR-ELISA over EB-EIA and optimal sensitivity using the TaqMan assay (Table 7.1). None of the RSD-free canes were found to contain *Cxx* using the EB-EIA which gave a positive result for only 9 of the 16 RSD-infected cane samples. The PCR-ELISA assay detected *Cxx* in all of the RSD-infected canes as well as 4 of the 16 putatively RSD-free canes. The TaqMan assay also detected *Cxx* in all of the RSD-infected canes and in14 of the 16 putative RSD-free. This result demonstrated that the TaqMan assay was more sensitive than the PCR-ELISA and confirmed the increased sensitivity of PCR-ELISA over EB-EIA.

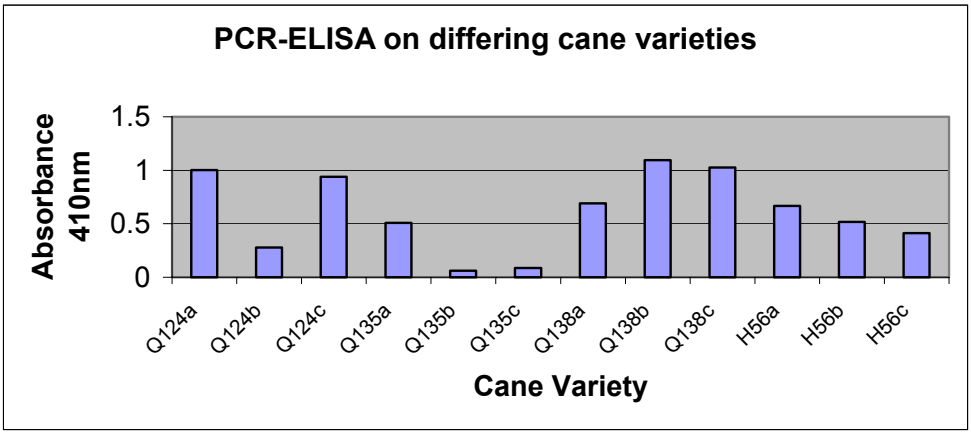

Figure 7.2 Variable *Cxx* titres in FVF of differing cane varieties as assessed using PCR-ELISA. Potential reasons for such variability include presence of *Taq* DNA polymerase inhibitors or differing levels of titre conferred by the phenotype of the variety.

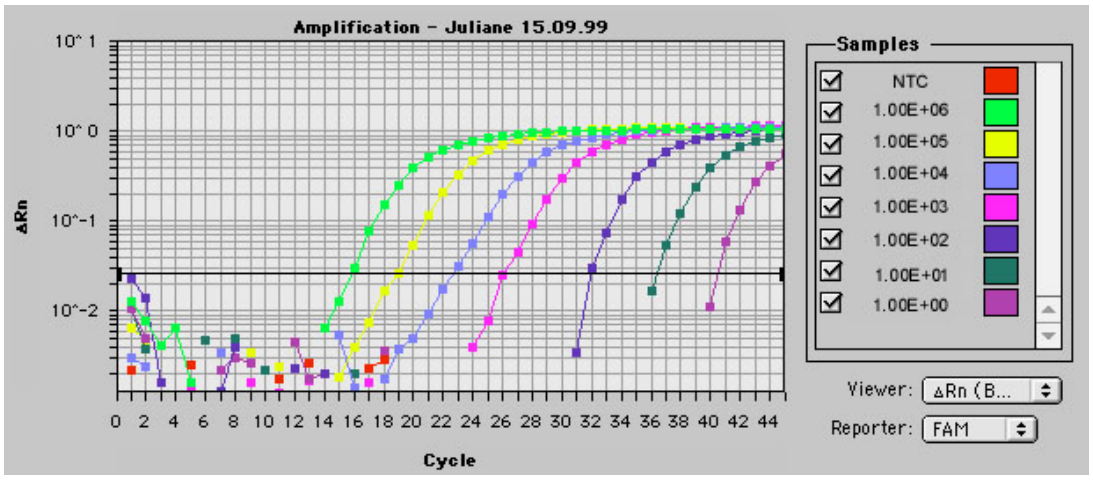

Figure 7.3 TaqMan amplification plots showing fluorescent output vs. thermal cycle number for serial 10-fold dilutions of pCLAVI in water (10^6 to 1 copies) as template for PCR. The TaqMan assay is capable of detecting a single template if present in the reaction.

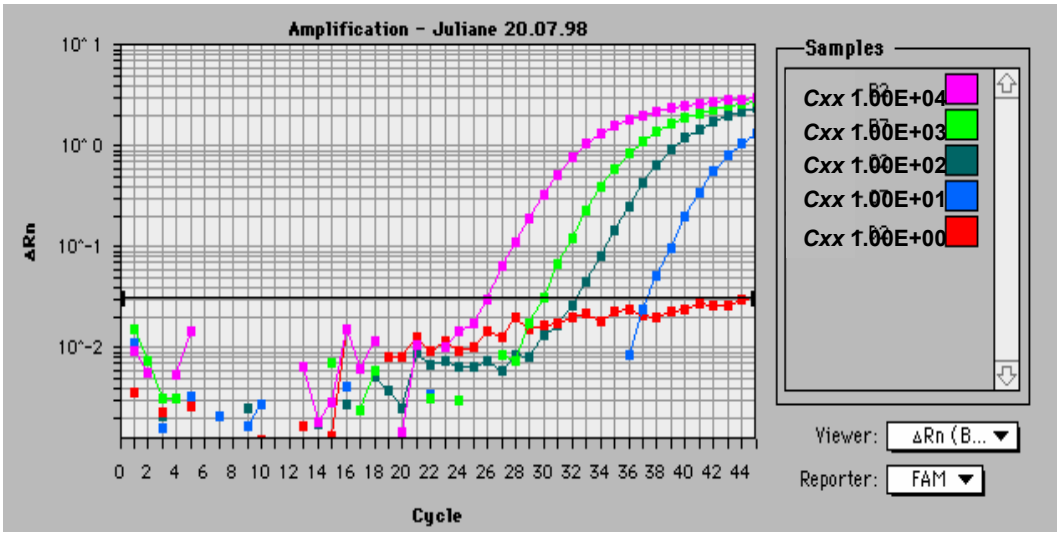

Figure 7.4 TaqMan amplification plots of 10-fold serial dilutions of purified *Cxx* in water (10^4 to 1 cell). The TaqMan assay is capable of detecting a single *Cxx* cell although this is affected by the likelihood of one cell being present in the microlitre volume sampled (no reaction was given by the 1 cell/ uL dilution in this experiment).

	Number of Samples Testing Positive	
Assay Method	**"RSD-Free" canes**	**"RSD-Infected" canes**
EB-EIA	0(16)	9(16)
PCR-ELISA	4(16)*	16(16)*
TaqMan	14(16)	16(16)

Table 7.1 Comparison of EB-EIA, PCR-ELISA and TaqMan for detection of *Cxx* in FVF samples collected from putatively "RSD-free" or putatively "RSD-infected" cane samples. The canes were grown in a RSD demonstration plot at BSES, Tully. The TaqMan assay detected *Cxx* template in all "RSD-infected" canes and 14 of the putative "RSD-free" canes. Values in parentheses indicate number of samples tested.

* Many of these PCR-ELISA values were in the "doubtful positive" range, i.e. near the limits of detection.

Table 7.2 presents a summary of the quantitative data obtained by the TaqMan analysis from the Tully RSD demonstration plots described in Table 7.1. The majority of putative RSD-free samples that tested positive contained very low titres of *Cxx*. These results suggest that low-level cross-contamination, most probably introduced during collection and/or transport of the samples, had occurred. These results illustrated the extreme sensitivity of the TaqMan assay and prompted a review of techniques for sample collection from sugarcane.

After this field trial the use of PCR-ELISA was discontinued as it was less sensitive, more laborious and offered less security for the validation of data at the limits of detection than the TaqMan assay for *Cxx.*

Size Groups (cells/μL)	**"RSD-Free" plot (cells/μL) (16 samples)**	**"RSD-Infected" plot (cells/μL) (16 samples)**
0	0,0	-
0-10	2, 3, 3, 4, 5, 5, 6, 7	-
11-100	20, 25, 63, 63	19, 20, 41, 85
101-1000	106	123, 136, 151, **202*, 331*, 750*, 984***
1001-10000	3357	**1154*, 3152*, 3724***
>10000	-	**11245*, 19823***

Table 7.2 Quantitative TaqMan data for analysis of samples from the Tully Demonstration plot pilot study (cf. Table 7.1). Concentration of *Cxx* cells present in cane sample FVF were deduced from a pCLAVI standard curve. Results are shown as number of *Cxx* cells in each 1μL sample assayed.
*** denotes samples testing positive to EB-EIA**

7.2.3 Field Trial 2: Samples from BSES Eight Mile Plains Pathology Farm

A further field study, directed towards finding alternative methods of sample collection and storage to avoid potential cross-contamination, was conducted at the BSES Eight Mile Plains Pathology Farm. Five putative RSD-free canes, five putative RSD-infected canes and five *Xanthomonas albilineans*-infected canes (RSD disease status unknown) were sampled. For each cane, FVF and pith tissue samples were collected into screw-capped, O-ring tubes and the samples were tested using EB-EIA and TaqMan. The results are summarized in Table 7.3.

There were four important outcomes from this field study. First, no *Cxx* was detected in any of the putative RSD-free canes or the *Xanthomonas*-infected canes using either EB-EIA or the TaqMan assay. This result suggested that the use of O-ring collection tubes assisted the avoidance of cross-contamination. Second, this study showed no cross-reactivity between the TaqMan *Cxx* assay and *Xanthomonas,* illustrating the specificity of the test. Thirdly, there was good correlation between the EB-EIA and TaqMan results for RSD-infected FVF samples, with TaqMan having the added advantage of providing quantitative data. Finally, the increased sensitivity of TaqMan allowed detection of *Cxx* in pith tissue samples. While the pith data did not correlate directly with the results seen for FVF (due to the gentle method used to release bacterial cells from pith without also releasing inhibitory cellular materials), it clearly demonstrated the potential to use samples other than FVF for routine testing by TaqMan.

	Plot #1 Putative "RSD-free"		Plot #2 Xanthomonas-Infected		Plot #3 Putative "RSD-Infected"	
Sample Number	***FVF* (cells/μL)**	**Pith tissue (cells/μL)**	**FVF (cells/μL)**	**Pith tissue (cells/μL)**	**FVF (cells/μL)**	**Pith tissue (cells/μL)**
1	0	0	0	0	1100	27
2	0	0	0	0	1700	24
3	0	0	0	0	210000	81000
4	0	0	0	0	260000	10000
5	0	0	0	0	1700000	40000

Table 7.3 Quantitative TaqMan data for *Cxx* in samples from a field trial at BSES Eight Mile Plains Pathology Farm. The purpose of this field study was to determine if O-ring tubes for collection and storage eliminated low-level cross-contamination seen in the Tully pilot study. Results are shown as number of *Cxx* cells in each 1μL sample assayed.

7.2.4 Field Trial 3: Samples from Nambour region

Larger scale field studies were conducted to test the robustness of the TaqMan assay and collection techniques. One such study, conducted in the Nambour region, involved collection from four cane plots of different varieties and variable RSD history. The samples consisted of **Plot 1:** putative RSD-infected Q138 cane; **Plot 2:** unknown RSD status Q146 cane; **Plot 3:** putative RSD-free Q154 cane and **Plot 4:** CP51-21B cane planted in a field with previously recorded RSD-infection but current status unknown. Samples of FVF and pith tissue were collected from ten cane stalks within each plot and assayed using EB-EIA and TaqMan.

Table 7.4 shows that no *Cxx* was detected in any sample from plot 2 (unknown RSD status) or plot 3 (putative RSD-free) using either TaqMan or EB-EIA. Both TaqMan and EB-EIA detected *Cxx* in 100% of canes from plot 1 (putative RSD-infected) with TaqMan revealing titres between 280 cells/μL and 29000 cells/μL for FVF and 1 cell/μL and 110 cells/μL for pith tissue (Table 7.4). For plot 4 (cane planted in a field with previously recorded RSD-infection) TaqMan found *Cxx* in 7/10 FVF samples (titres ranging from 600 cells/μL to 4800 cells/μL) and 6/10 pith samples (titres ranging from 1 cell/μL to 77 cells/μL). In comparison, EB-EIA found *Cxx* in 6/10 FVF samples and 6/10 pith tissue samples. Importantly, the absence of *Cxx* in samples from plots 2 and 3 demonstrated that there was no cross-contamination of samples during collection and transportation. This was a particularly interesting result as the equipment used to collect xylem exudate from plots 2 and 3 had previously been used to collect RSD-infected FVF from plot 1 canes.

To test the reproducibility of the *Cxx* TaqMan assay as well as assess stability of FVF samples during storage conditions, the same pith samples from the Nambour field trial were retested after three freeze/thaw cycles during three months storage at –20^{o}C. The results (Figure 7.5) indicate that the TaqMan assay for *Cxx* was highly reproducible (within 1 PCR cycle) and showed DNA template from *Cxx* in cane FVF was stable during multiple freeze/thaws.

Sample #	Plot #1	Plot #2	Plot #3	Plot #4

from plot	(Q138VR) "RSD-Infected" (cells/μL)		(Q146VR) "Unknown" (cells/μL)		(Q154F) "RSD-free" (cells/μL)		(CP51-21B) "Previous Infection" (cells/μL)	
	FVF	Pith tissue	FVF	Pith tissue	FVF	Pith tissue	FVF	Pith tissue
1	29000	n/a	0	0	0	0	880	77
2	4600	1	0	0	0	0	0	0
3	3000	11	0	0	0	0	0	0
4	6100	9	0	0	0	0	600	20
5	1700	3	0	0	0	0	4800	15
6	5300	1	0	0	0	0	1600	0
7	5500	110	0	0	0	0	600	12
8	280	13	0	0	0	0	0	0
9	12000	1	0	0	0	0	800	14
10	3500	110	0	0	0	0	1800	1

Table 7.4 Quantitative TaqMan data for *Cxx* in samples from field material grown in the Nambour region. Results are shown as number of *Cxx* cells in each 1μL sample assayed. Data for one sample (Q138VR-1) is not available (n/a) as it returned an erroneous result due to spurious fluorescence signal produced during PCR amplification. This sample was retested. Importantly, no *Cxx* cells were detected in samples from plots 2 and 3 using TaqMan. These samples were collected immediately following plot 1 (RSD-infected) indicating that the use of screw-capped, O-ring tubes is effective in the prevention of cross-contamination during sampling.

Results from this experiment are presented to illustrate a useful feature of the Model 7700 Sequence Detection System when used for the TaqMan assay. The "multicomponent view" displays the component dye signals that contribute to the composite signal for a selected reaction tube. During multicomponent analysis, an algorithm resolves the contribution of each individual dye to the spectral data collected during PCR. In this way, it is possible to distinguish a "false positive" result by inspection of the pattern of emission of each fluorescent dye in the tube during amplification.

As an example, one of the samples from plot 2 (Q146VR-6) appeared RSD-infected when first tested in June 1999 (Table 7.4). However, using the multicomponent analysis function of the software, this result was shown to be a "false-positive". Figure 7.6 shows the multicomponent analysis view for sample Q146VR-6. The emission of the reporter (FAM) and quencher (TAMRA) dyes remain parallel indicating that no cleavage of probe has occurred and therefore there has been no amplification, indicating the absence of a *Cxx* target sequence (the false positive result was caused by excessive signal noise generated during the first 15 PCR cycles). In contrast, Figure 7.7 shows a multicomponent analysis of a real amplification result. Note that as amplification proceeds, the emission for the reporter (FAM) increases as the quencher (TAMRA) decreases. This result indicates that there has indeed been cleavage of probe and therefore amplification of the required product.

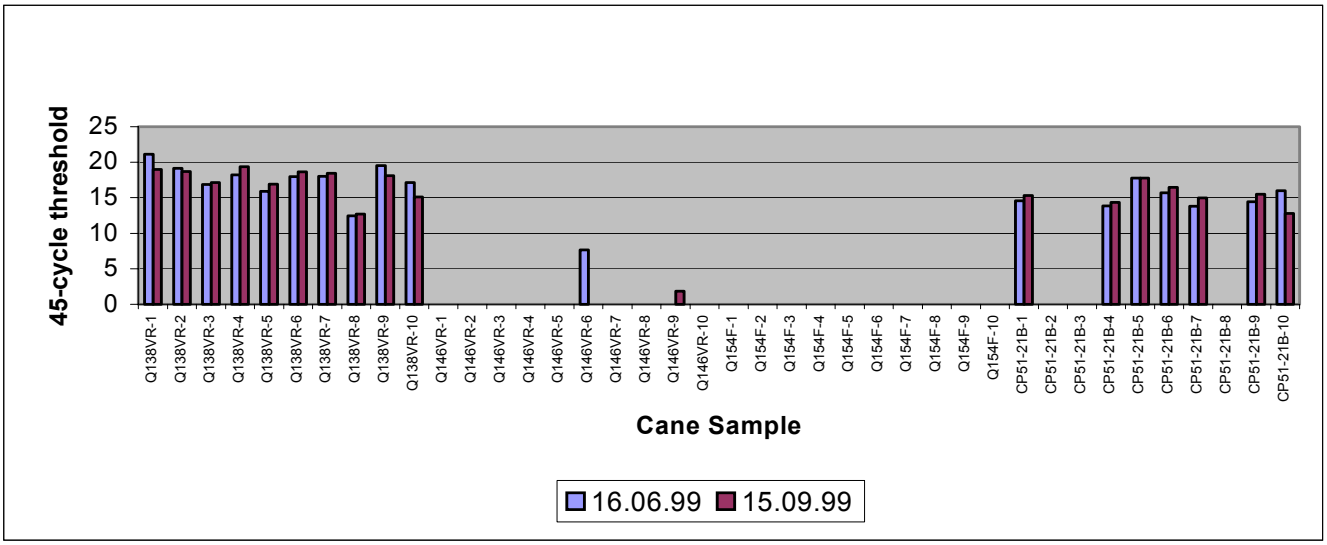

Figure 7.5 Reproducibility of the TaqMan assay for samples collected from Nambour field trial (cf. Table 7.4). FVF samples were subjected to three freeze-thaw cycles during storage at –20^{o}C for 3 months after the assay presented in Table 7.4. Results were highly reproducible (within 1 PCR cycle) and showed no evidence of sample degradation. One sample, gave a "false positive" result in the previous assay (Plot#2, Sample #6 (Q146VR-6), Table 7.4) which was confirmed as negative using multicomponent analysis data (See Figure 7.6). Retesting in this experiment confirmed the negative result.

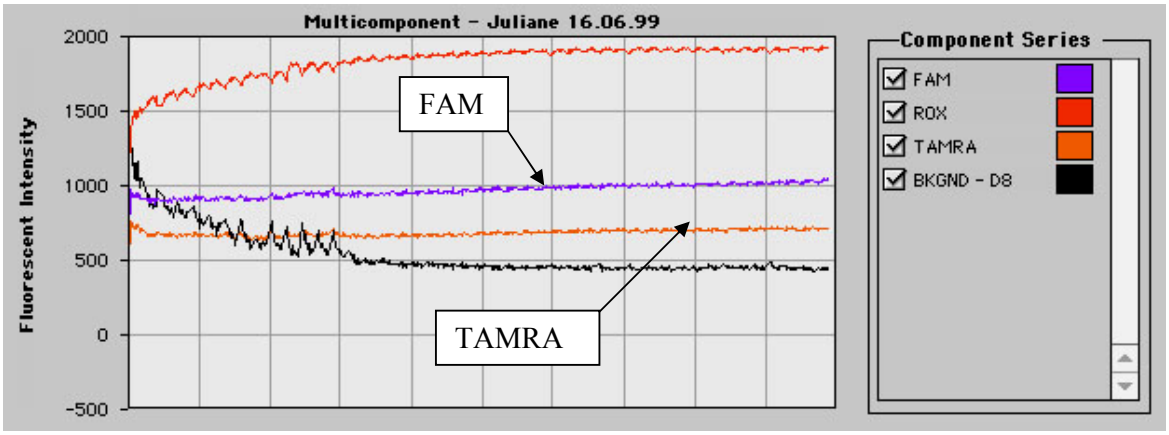

Figure 7.6 Multicomponent analysis of Nambour field sample (FVF sample, Plot #2, sample #6 [Q146VR-6] in Table 7.4) confirming the result as a false positive. The emission of the reporter (FAM) and quencher (TAMRA) dyes remain parallel indicating that no cleavage of probe has occurred and therefore there has been no amplification. The Background (black) and Internal Reference dye ROX (red) deviate from linearity indicating abnormal reaction signals.

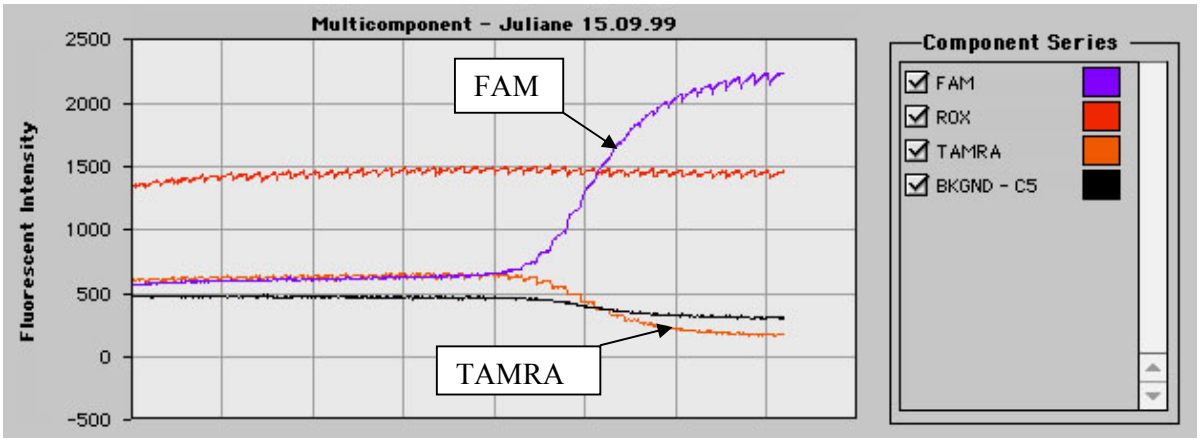

Figure 7.7 Multicomponent analysis of a Nambour field sample containing *Cxx* (FVF sample, Plot#1, sample #5 [Q136VR-5] in Table 7.4). Note that as amplification proceeds, the fluorescence emission for the reporter (FAM) increases as the quencher (TAMRA) decreases. This result indicates that there has indeed been cleavage of probe and therefore amplification of the desired PCR product.

7.2.5 Comparison of TaqMan and EB-EIA in RSD resistance trial

A RSD resistance trial was held in the first half of 2000, in which breeding and other germplasms representing many different genetic lines were tested by inoculation with *Cxx*, and subsequently sampled in April and June to obtain FVF for analysis. Levels of RSD infection were assessed in each FVF sample using both the TaqMan and EB-EIA assays. Details of experimental setup etc. are not presented here, as the main objective from the view of Project UQ024 was to compare the results of these two assay systems for diverse samples representing a range of germplasms.

FVF samples collected in April 2000: A total of 58 FVF samples were assayed by both TaqMan and EIA; the data are presented as a scatter plot (Figure 7.8). Of these samples, 36 tested negative both by EB-EIA (zero absorbance) and TaqMan (no amplification after 45 cycles), and appear as a single point on Fig. 7.8. A further four samples that tested negative by EB-EIA (absorbance values of 0, 0, 0, 0,03, i.e. <0.05) tested positive by TaqMan and gave a low titre of <10 *Cxx* cells/µL (C_t 37-40 cycles). Another four samples that were rated doubtful by EB-EIA (absorbance range 0.05-0.15) all tested positive by TaqMan (C_t values ranging from 30-36 cycles, equivalent to ~1,000-10 *Cxx* cells/µL respectively). The remaining 14 samples gave clearly positive reactions with both the TaqMan and EB-EIA systems.

An overall positive correlation was obtained between EB-EIA (as absorbance) and TaqMan (as C_t value, with negative values given as 45 cycles) using the data as displayed in Fig 7.8. The TaqMan assay was particularly useful in demonatrating the presence of RSD in FVF samples with low *Cxx* bacterial titres. However, the R^2 value of 0.62 indicates some variability in the value of *Cxx* titre obtained by these two assay systems across all samples tested.

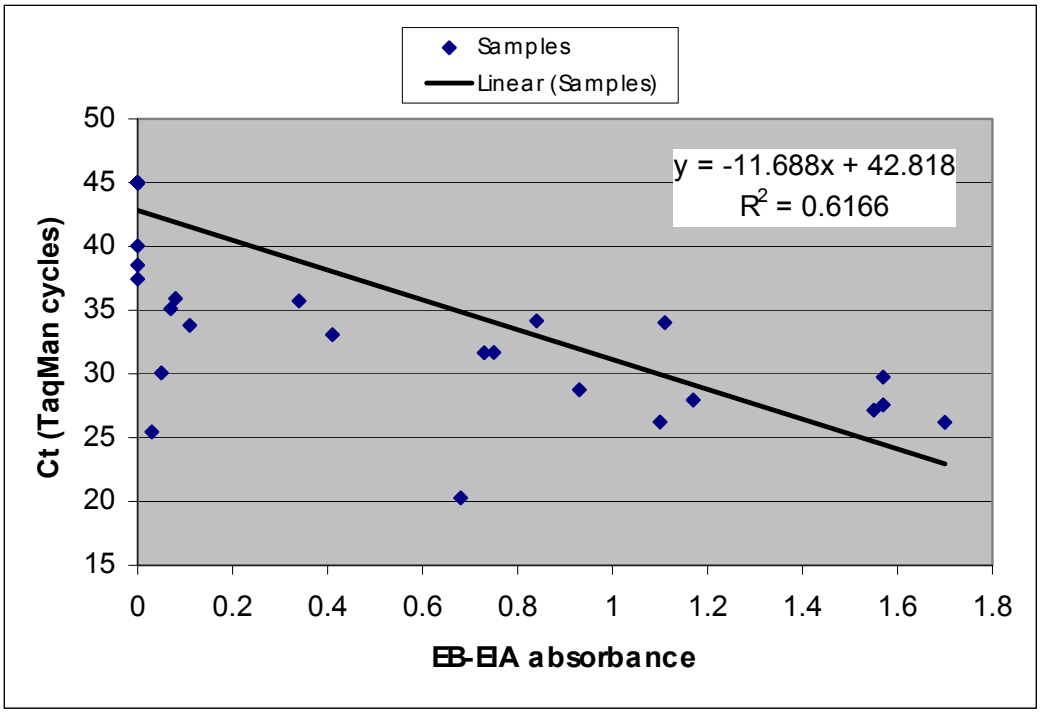

Figure 7.8. Scatter plot showing correlation between TaqMan and EB-EIA assays for *Cxx* in FVF fluid collected from 58 cane samples as part of a *Cxx*-resistance trial of various sugarcane varieties.

FVF samples collected in June 2000: A total of 281 FVF samples were assayed by both TaqMan and EB-EIA (Figure 7.9). Since all of the cane plants tested had been inoculated with *Cxx*, some level of RSD infection was possible in any of the FVF samples collected. The TaqMan data in Fig. 7.9 are expressed as the $\log_{10}$ of cells/µL to show the wide dynamic range of titres obtained.

Of the 281 FVF samples tested, 71 tested negative with both EB-EIA and TaqMan, indicating either lack of RSD infection, or that the sampling protocol or infection level did not allow provide a sufficiently high bacterial titre in FVF for TaqMan assay to detect *Cxx*. The TaqMan assay gave a positive reaction (range1-400 cells/µL, average 19 cells/µL) with a further 34 samples which were rated negative with EB-EIA (Absorbance <0.05). Another 24 samples which tested doubtful with EB-EIA (Absorbance 0.05-0.15) gave a clear reaction with the TaqMan assay (8-156 cells/µL, average 46 cells/µL).

The remaining FVF samples tested positive by both EB-EIA and TaqMan, and in no case did the TaqMan assay fail to detect *Cxx* in samples that tested positive with EB-EIA (Absorbance >0.15). However, although the TaqMan assay showed a positive correlation with EB-EIA over the whole 281 samples tested, the correlation coefficient again was not particularly strong (R^2 = 0.64). It can therefore be concluded that the TaqMan assay is indeed more sensitive than the EB-EIA assay and can reliably detect low levels of *Cxx* cells in FVF collected from *Cxx*-inoculated cane under controlled field conditions. Further experiments are required to determine whether *Cxx* builds up to higher levels in canes exhibiting such low-level RSD infections over time, and to determine the cause of the relatively poor correlation in quantitative titre obtained by these two assay methods.

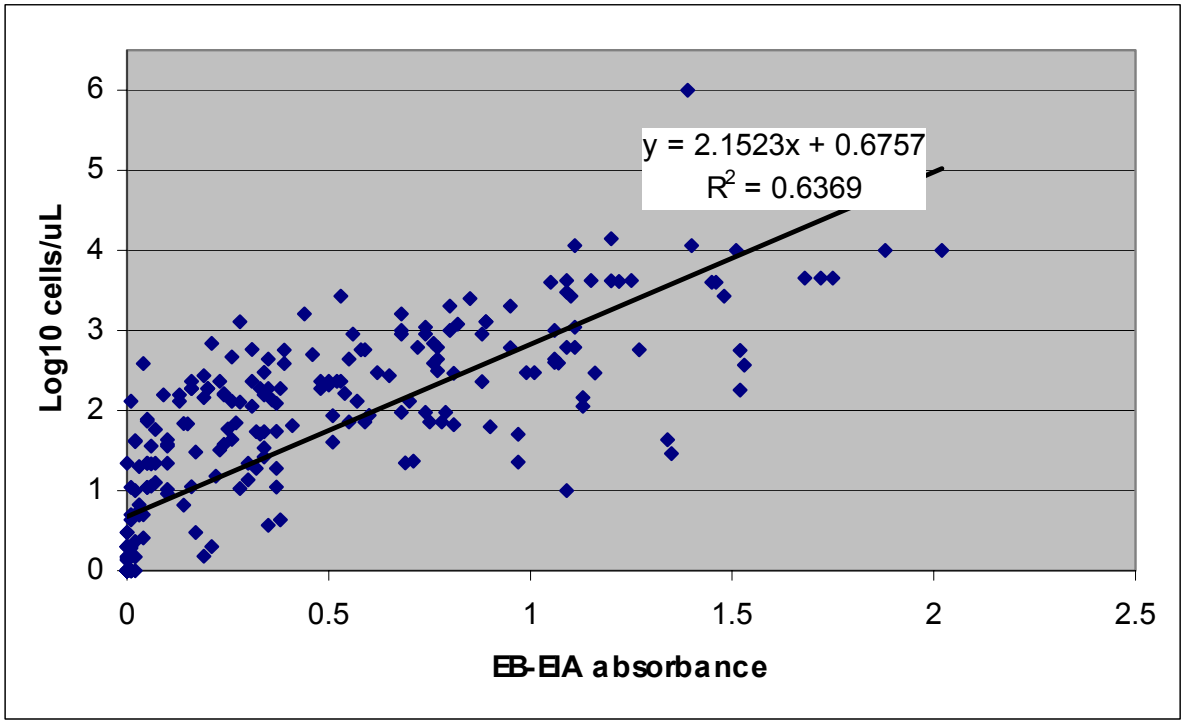

Figure 7.9. Scatter plot showing correlation between TaqMan and EB-EIA assays for *Cxx* in FVF fluid collected from 281 cane samples as part of a *Cxx*-resistance trial of various sugarcane varieties.

7.2.6 Alternative cane sampling strategies using the TaqMan *Cxx* assay

During the course of development and validation of the TaqMan assay, alternative sampling strategies for *Cxx* were investigated to streamline sample collection and decrease potential for cross-contamination. It was envisaged that the increased sensitivity and specificity conferred by the TaqMan system would allow samples such as cane juice, leaf segments and pith tissue to be assayed reliably.

Collection of cane juice is much simpler and cleaner than FVF. Cane juice may be obtained by bending or crushing the cane stalk or "tapping" the stalk using an implement designed to channel the juice directly into a collection vessel. However, cane juice contains a much higher concentration of cellular materials that could act as inhibitors of PCR, the most abundant of which is sucrose. The effect of cane juice on PCR can be seen in Figure 7.10. This figure illustrates a comparison of PCR efficiency of samples of FVF and cane juice taken from the same plants. Samples of FVF from *Cxx*-infected canes gave the expected exponential amplification curves in the TaqMan assay. In comparison, cane juice samples collected from the same plants showed considerably slower amplification kinetics, characteristic of PCR inhibition. Multicomponent analysis of data from the cane juice samples indicated that the PCR amplification curves were real (results not shown), consistent with *Cxx* indeed being present in the reaction tubes. However, for reliable detection of Cxx in routine cane juice samples, the problem of inhibition must be overcome. Attempts to overcome inhibition of PCR by the addition of polyvinylpyrrolidone (PVP) to the reaction mixtures have thus far been unsuccessful. PVP is a soluble, high molecular weight compound reported to improve stability of enzymes by binding impurities, and has been shown to reduce Taq inhibition during PCR for gel-based detection of *Cxx* (Fegan et al., 1998). However, it appears that PVP is unable to overcome Taq inhibition caused by the intracellular materials present in cane juice. It is possible that by concentrating and washing particulate matter in cane juice prior to PCR, e.g. by centrifugation or filtration, such inhibitory materials might be removed from cells of *Cxx* bacteria prior to assay by PCR. However, time did not permit such experiments to be conducted.

Results from "Field Trial 2" (BSES Eight Mile Plains Pathology Farm) showed that, in addition to FVF, pith tissue samples could be used to screen for *Cxx*. Although levels of detection were decreased about 10-100 fold, the potential to detect *Cxx* in other cane sample types was clearly demonstrated using the highly sensitive TaqMan assay. During this field trial, samples of leaf tissue were also tested. However, *Cxx* was detected in only one of the five putative RSD-infected canes using leaf material. Leaf tissue was processed in the same manner as pith tissue (ie. soaked in sterile water to allow any *Cxx* present to exude into the medium). It is possible that the inclusion of a simple homogenisation step could enable the detection of *Cxx* in leaf samples and make it possible to avoid the complicated procedures required to collect FVF. Due to time constraints, such experiments were not attempted.

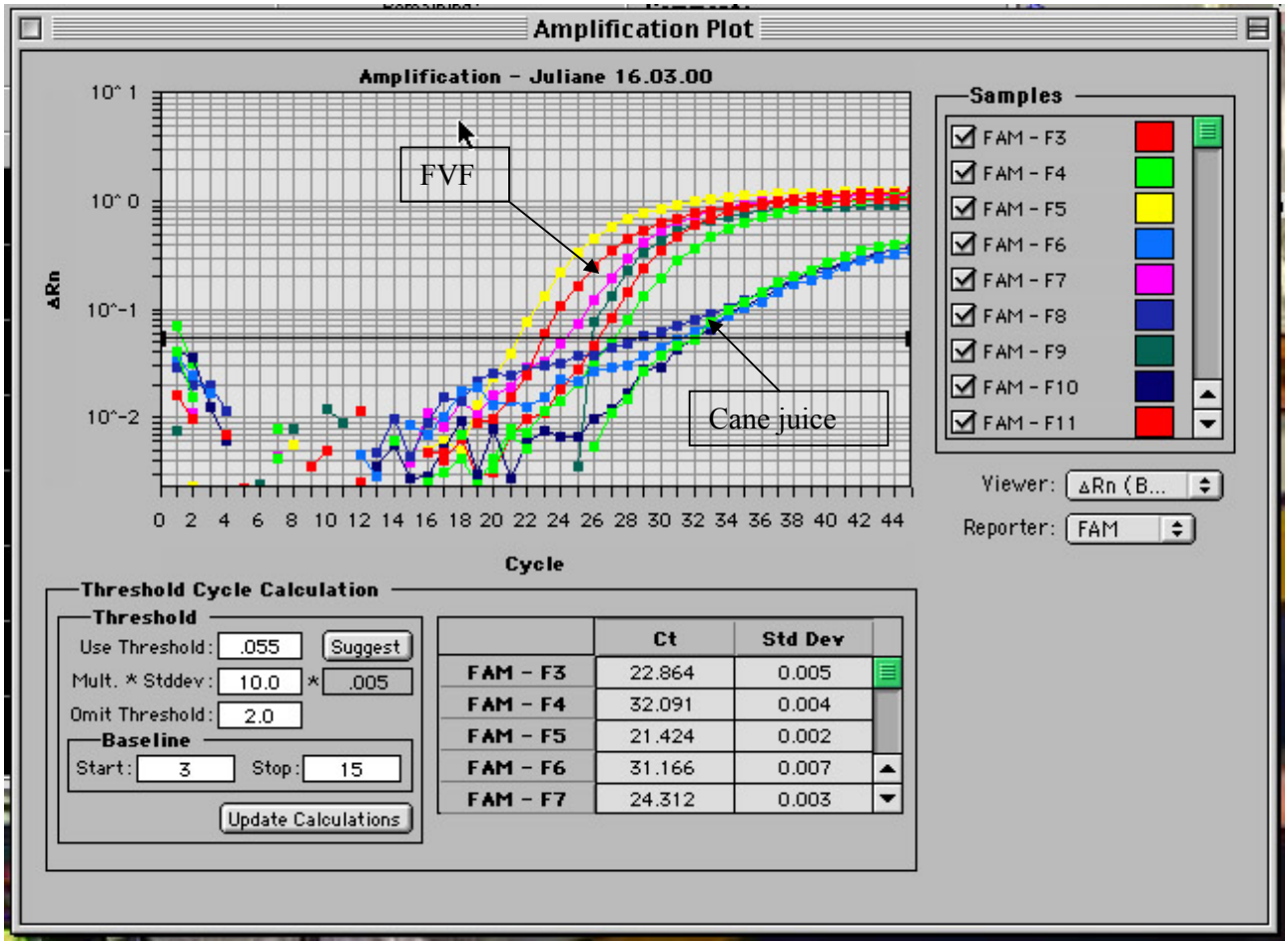

Figure 7.10 Comparison of TaqMan amplification plots detecting *Cxx* in FVF and cane juice. Samples of FVF and cane juice were taken from the same plants and tested in parallel. Amplification plots of *Cxx* in FVF samples showed exponential growth curves while cane juice samples showed slow amplification kinetics characteristic of PCR inhibition.

7.2.7 Quarantine applications of the *Cxx* TaqMan assay

The reliability of the TaqMan assay for detecting *Cxx* was further tested using quarantine cane samples. These sample types provide a challenge for detection of *Cxx* by EB-EIA as immature canes often contain little FVF or are nodal sections from cut cane ready for planting. Since the *Cxx* TaqMan assay requires a sample size of only 1μL and has been shown capable of detecting *Cxx* in sugarcane pith tissue, it was suggested that TaqMan may be appropriate for testing difficult samples such as these.

Initially, the *Cxx* TaqMan assay was used to screen 15 varieties of BSES quarantine canes marked for export. Segments from immature canes were centrifuged to collect FVF for testing. The TaqMan assay showed that none of these samples contained *Cxx*, reassuring the hygiene of the export material.

In March 2000, the TaqMan *Cxx* assay was used to screen overseas accessions held in quarantine at BSES, Indooroopilly. A total of 68 samples were tested including isolates from Reunion Island, South Africa, Taiwan, US, Brazil, Columbia, Mauritius and Hawaii. Sugarcane pith tissue extracts (obtained by gently crushing pith tissue in sterile water) were obtained directly from BSES. As a result of this testing, one sample originating from the US (CP88-1409 from Florida), was found to contain *Cxx* cells at a level of 150 cells/μL (150,000 cells/mL). This level of infection is below the limit of detection of the EB-EIA (approximately 1,000,000 cells/mL) for *Cxx* and would probably not have been detected without the increased sensitivity of the TaqMan assay. In response to this finding, BSES Indooroopilly were notified immediately and the cane from which the sample originated was kept under surveillance by BSES in the containment glasshouse for retesting at a later stage.

The clones included in the above study were grown to maturity for retesting, and standard FVF samples were collected for assay by BSES staff. Clone CP88-1409, which earlier tested positive to *Cxx* by TaqMan, was not confirmed as positive by EB-EIA or PCM. TaqMan assays of the FVF from clone CP88-1409 also tested negative. Four other clones that had previously tested negative by TaqMan, now tested positive by EB-EIA and PCM. However FVF from accessions other than clone CP88-1409 were not assayed by TaqMan (Project UQ024 had now terminated).

The above inconsistencies indicate the difficulty of using any early testing system for quarantined clones, as a thorough validation process must be designed and tested over time, including growing test canes to maturity and taking samples during cane development, before such an early test system could be considered reliable. For example, *Cxx* infections could be latent or unevenly distributed in some accessions, and if titres are low in billets or immature cane, *Cxx* might be missed by any laboratory assay even if sufficient FVF could be collected. Growing clones to maturity before assessing visible symptoms and testing by the three methods available (TaqMan, EB-EIA and PCM) must be considered the benchmark before any laboratory-based test could be used as a sole test for RSD in quarantine germplasm.

The above result highlights the need for thorough validation of any test to be used for quarantine screening. The extreme sensitivity of the PCR-based TaqMan assay compared to EB-EIA or PCM should prove useful in this respect, although even an assay that can detect a single bacterial cell in a 1 μL FVF sample (which cannot represent the whole billet or plant) may be insufficient in some situations. It is unfortunate that after development of the TaqMan assay and validation with field material (where an occasional low titre missed by a laboratory test is not crucial due to multiple sampling from a field), Project UQ024 was coming to an end when more exacting procedures were necessary to validate the assay for quarantine screening and similar purposes e.g. resistance trials (cf. Section 7.2.5).

7.3 Analysis of sequence variability within FDV

As no prior studies had been carried out on the strain diversity of FDV in Australia, it was necessary to examine sequence variability prior to the development of diagnostic tests for FDV. Samples from two geographically separate locations were used to examine the potential variability of the target sequence. FDV-infected tissue was collected from the BSES Eight Mile Plains Pathology Farm and from a commercial sugarcane farm in Harwood, NSW. FDV dsRNA was extracted from gall tissue using the rapid release protocol (Thomson and Dietzgen, 1995) and the required fragment was amplified using primers FDV-7F and FDV-7R (Smith *et al.,* 1994) and the Titan RT-PCR kit (Roche).

A 450bp amplification product was obtained from both the Eight Mile Plains and Harwood FDV samples. These products were ligated into the pGEM-T vector (Promega) and three clones of each were sequenced in forward and reverse directions using the universal sequencing primers T7 and SP6. Despite minor sequence variations (two nucleotides substitutions in one clone, and one substitution in each of two other clones), the exact consensus sequence was given by one or more clones derived from each site, and it is possible that some or all of the observed variation was caused by errors during reverse transcription or PCR during clone production. This may indicate relatively little sequence variation in Australian isolates of FDV although a more extensive sequence study incorporating a wider range of isolates would need to be undertaken to confirm this.

The consensus FDV sequence obtained using the Eight Mile Plains and Harwood isolates was used for the design of oligonucleotide primers and probes for a PCR-ELISA and TaqMan assay.

7.4 Development of an RT-PCR-ELISA for FDV

An RT-PCR-ELISA assay was developed to detect FDV in sugarcane and planthoppers. The assay is specific to one of the 1.7kb dsRNA linear segments of FDV, using primers FDV-7F and FDV-7R (Smith *et al.,* 1994) and an internal hybridisation probe, FDV-bio-7, designed based on sequences described in section 7.3 (Pickering, 1997).

Sensitivity of the assay was assessed using a 10-fold dilution series of purified FDV dsRNA ranging from $2x10^7$ FDV genomes per reaction (2.9ng) to 2 FDV genomes per reaction (290ag). Products were amplified using the Titan RT-PCR kit (Roche) and detected using the PCR-ELISA Detection Kit (Roche) and the oligonucleotide probe FDV-bio-7. Detection on agarose gel was also carried out for comparison.

Comparing detection of duplicate 5µL aliquots, only the most concentrated sample ($2x10^7$ FDV genomes per reaction) was detected via agarose gel electrophoresis. However, the FDV RT-PCR-ELISA detected from $2x10^7$ down to $2x10^4$ FDV genomes per reaction. These results indicate that the RT-PCR-ELISA was approximately1000 times more sensitive than gel detection of RT-PCR products.

7.5 Development of a TaqMan assay for FDV

A TaqMan assay was designed to one of the 1.7kb dsRNA linear segments of the FDV genome. Specific primers FDV-727F and FDV-Tq-7R amplified a 236bp product that was subsequently detected using the internal hybridisation probe FDV-FAM-7.

The EZ-RT-PCR kit (Applied Biosystems), which is based on the dual reverse-transcriptase and DNA polymerase activities of the thermostable *Tth* DNA polymerase, was used to develop the

assay. This system was chosen to overcome difficulties posed by the highly stable dsRNA genome of FDV. An initial denaturation step at 95^{o}C is necessary to open the dsRNA and provide access to the oligonucleotide primers prior to dropping temperature to 60^{o}C to start the reverse transcriptase reaction. This relatively high reverse transcriptase temperature also helps to avoid potential problems with RNA secondary structure.

Sensitivity of the assay was assessed using a 10-fold dilution series of purified FDV dsRNA ranging from 1x10^{7} FDV genomes per reaction (1.5ng) to 1 genome per reaction (150ag). The TaqMan assay was found to be sensitive reliably down to 10 FDV genomes per reaction (1.5fg of purified dsRNA) and possibly a single genome per reaction (150ag of purified dsRNA; this single genome sensitivity requires confirmation).

The FDV TaqMan assay was found to be at least 10^{6} times more sensitive than detection of PCR products by agarose gel electrophoresis, and 10^{3} times more sensitive than RT-PCR-ELISA.

7.6 Detection of FDV in sugarcane and planthoppers

The optimised RT-PCR-ELISA and TaqMan assays for FDV were first tested for sensitivity and specificity with a variety of samples including: (1) sugarcane leaf gall tissue, (2) healthy, glasshouse-grown sugarcane, (3) planthoppers taken from an FDV-infected sugarcane plot at the Eight Mile Plains BSES Pathology Farm and (4) FDV-free planthoppers taken from a sugarcane crop in Tully (due to regional quarantine, Tully is currently free of FDV). A negative control using water as template was also included.

The RT-PCR-ELISA for FDV detected high levels of FDV in sugarcane leaf gall tissue, and in planthoppers collected from an FDV-infected plot. ELISA Absorbance values for both of these samples were greater than those obtained previously for the purified FDV standard containing 2x10^{6} FDV genomes per reaction. The FDV-free sugarcane and planthopper samples gave readings comparable to the negative control. These results also demonstrated that the PCR-ELISA was specific for FDV and did not show any cross-reactivity with either sugarcane or planthopper DNA.

Similarly, using the TaqMan assay for FDV, reporter fluorescence was only detected in the sugarcane leaf gall sample and for the planthoppers collected from an FDV-infected plot. No fluorescence was recorded for the FDV-free sugarcane or planthopper DNA, demonstrating the specificity of the assay for FDV in the presence of nucleic acids from plant host or insect vector.

7.6.1 Determination of spatial distribution of FDV within sugarcane leaf tissue

Having demonstrated the ability of both the RT-PCR-ELISA and TaqMan assays to detect FDV present in sugarcane and planthopper nucleic acid extracts, the comparative sensitivity of these assays was tested to investigate two important issues related to FDV: (1) determination of the spatial distribution of FDV within a sugarcane leaf, and (2) screening of a planthopper population to determine incidence of FDV infection and the likelihood of transovarial transmission.

The first definitive symptoms of FDV are white, raised galls along the leaf midrib. FDV has been found in high concentrations in these galls (Rohozinski *et al.,* 1981). Until now, distribution of FDV throughout the sugarcane leaf has not been studied using sensitive molecular-based techniques. For quarantine screening, such tests need to be able to detect FDV in cane which has not yet developed visual symptoms.

The RT-PCR-ELISA and TaqMan assays were compared for their ability to detect FDV in various sites in FDV-infected sugarcane leaves. The following materials were examined: (1) FDV-free control leaves from glasshouse-grown plants, (2) an asymptomatic leaf taken from an FDV-infected cane plot, and (3) two leaves, A and B, each showing gall symptoms. Spatially separate leaf portions were excised from four sites within each of these leaves as shown in **Figure 7.9[7.11]**. The sample sites chosen were (#1) leaf midrib tissue (gall tissue taken if present), (#2) a site further along the same vascular tissue, (#3) leaf tissue adjacent to site #1, and (#4) leaf tissue adjacent to site #2. Crude nucleic acid extracts were made using the method of Thomas and Dietzgen (1995; details of this method can be found in Appendix 3)**.**

Using the RT-PCR-ELISA, samples taken from the gall tissue of the two leaves showing FDV symptoms (leaves "A" and "B", Figure 7.12) gave high OD readings confirming the presence of FDV. Samples adjacent to the galls (sites #2 and #3) of one of these leaves (FDV-infected leaf "A") also gave positive results indicating the presence of FDV, although the OD values were lower than those from the gall samples. All other samples gave OD values less than or similar to background for the glasshouse-grown FDV-free controls and therefore tested negative for FDV.

Figure 7.13 shows the results of TaqMan assays for the same sugarcane leaf samples assayed by RT-PCR-ELISA (see above). The TaqMan assay clearly demonstrated the presence of high viral titres in the three leaves collected from the FDV-infected plants. The samples taken from gall tissue in FDV-infected leaves A and B showed the highest titre of FDV as expected. Appreciable amounts of FDV were detected in most of the samples taken from asymptomatic sites in leaves A and B which exhibited typical FDV galls (the only exception was the sample taken from site #2 in infected leaf A). FDV was also detected in all four samples from the asymptomatic sugarcane leaf taken from a plant grown in the FDV-infected plot, despite the same crude extract from these four samples all testing negative by RT-PCR-ELISA. As expected, no FDV was detected in the putatively FDV-free, glasshouse grown sugarcane control.

These results clearly demonstrate the improved sensitivity that TaqMan offers over the RT-PCR-ELISA for FDV detection. The TaqMan assay should prove useful for further studies of distribution and movement of FDV throughout the sugarcane plant.

7.6.2 Detection of FDV in the planthopper vector *Perkinsiella saccharicida*

The planthopper *Perkinsiella saccharicida* is the only known vector of FDV present in Australia. Previous experiments using serological methods have shown these insects to be inefficient vectors (Francki *et al.,* 1986) and it has not been shown previously whether the virus is transmitted transovarially from parent to progeny. To determine whether transovarial transmission occurs in *Perkinsiella,* detection of FDV in newly emerged nymphs was attempted via RT-PCR-ELISA and the TaqMan FDV assay.

Fifteen newly emerged nymphs, raised from eggs laid in infected sugarcane leaf tissue, were pooled into four batches of 2-3 nymphs and total nucleic acid extracts were prepared using the method of Robertson *et al.,* 1991 (Appendix 3). These samples and "no template" controls were assayed both the TaqMan assay and a nested RT-PCR-ELISA protocol (two rounds of PCR, the template of the second round was the product from 30 cycles of the first round).

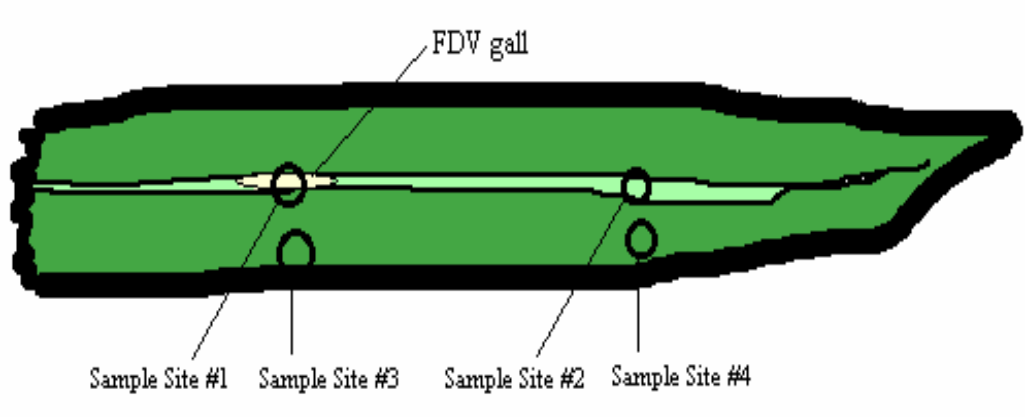

Figure 7.11 Schematic diagram of a sugarcane leaf showing the four sample sites used in the determination of FDV spatial distribution in sugarcane.

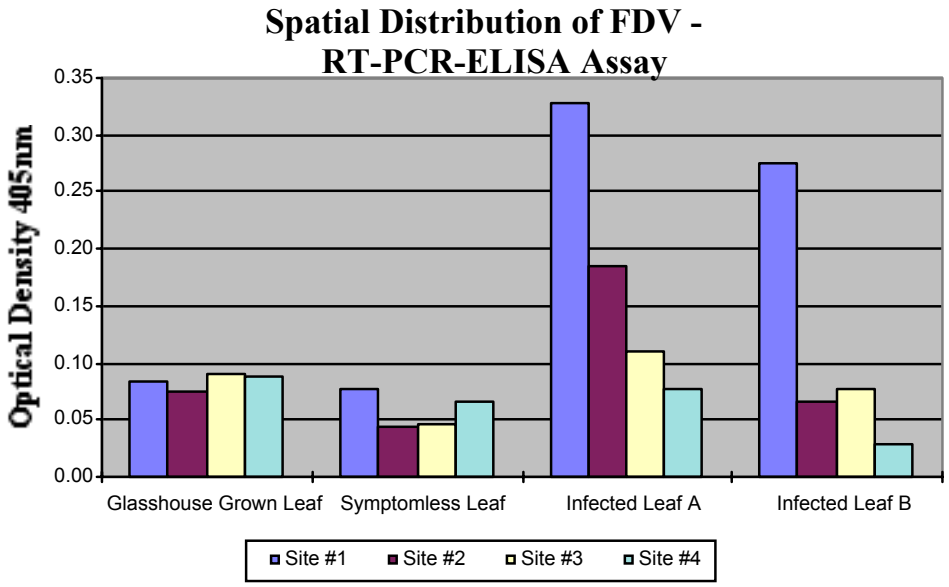

Figure 7.12 Determination of spatial distribution of FDV in sugarcane using RT-PCR-ELISA. Samples taken from the gall tissue (site #1) of infected leaves A and B gave high OD readings confirming the presence of FDV. Samples from site #2 and site #3 of infected leaf A also gave positive results indicating the presence of FDV, although at a lower level. All remaining sites gave OD values less than or similar to the glasshouse grown sugarcane indicating FDV was absent.

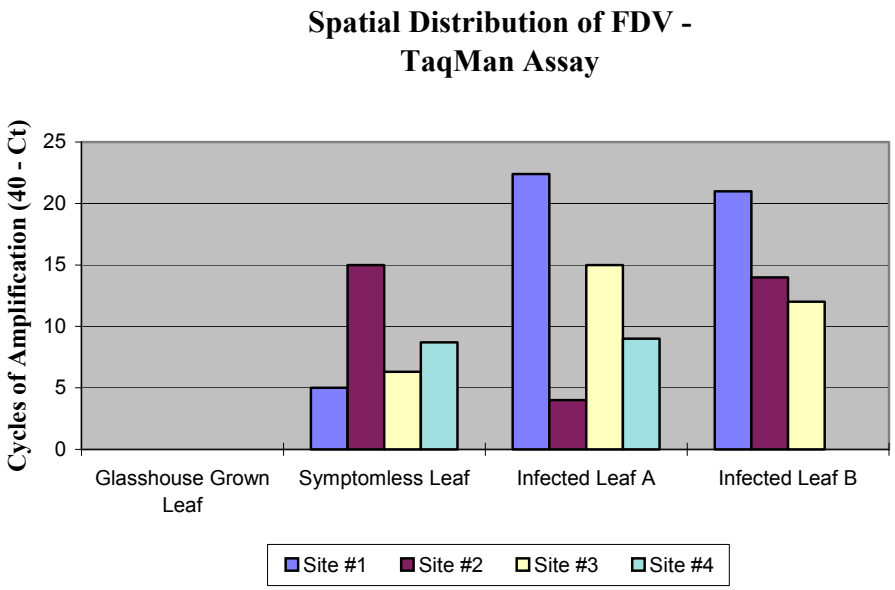

Figure 7.13 Determination of spatial distribution of FDV in sugarcane using the FDV TaqMan assay. Results are presented as (40 cycles – CT) to simplify presentation. FDV was found to be present in all samples except site #4 for infected leaf B. Interestingly, FDV was detected at all four sample sites in the asymptomatic sugarcane leaf tissue despite the same crude extract from these four samples testing negative in the RT-PCR-ELISA assays.

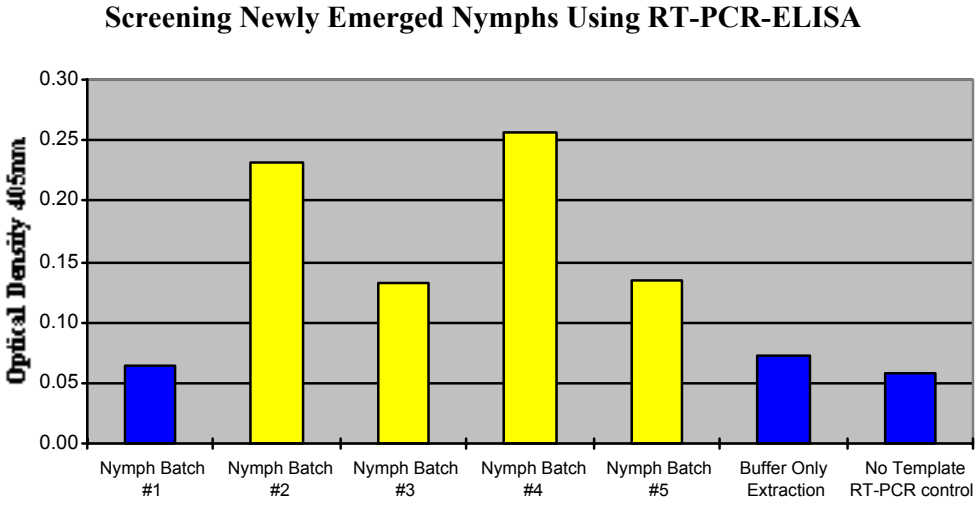

Figure 7.14 Detection of FDV in planthopper nymphs using nested RT-PCR-ELISA. FDV was detected in nymph batch numbers 2, 3, 4 and 5. Nymph batch number 1 showed OD levels similar to extraction and PCR controls indicating that no FDV was present. Interestingly, using the TaqMan assay, FDV was detected in all batches of nymphs (results not shown), including batch number 1 which tested negative for FDV using RT-PCR-ELISA.

Nymph batch numbers 2, 3 and 4 were positive for FDV using RT-PCR-ELISA, while batch number 1 gave the same background result as the "no template" controls and hence tested negative to FDV (Figure 7.14). It is not known how many of the individual nymphs within each positive batch contained FDV. Using the TaqMan assay, FDV was detected clearly in all batches of nymphs (results not shown).

These results demonstrate the increased sensitivity of TaqMan over RT-PCR-ELISA and the ability to detect FDV in newly emerged nymphs. However, this result does not unequivocally demonstrate that FDV is present in the nymphs due to transovarial transmission. It cannot be excluded that the newly emerged nymphs had opportunity to feed on (or accrete debris from) infected tissue surrounding the oviposition sites from which they hatched. However, we believe it is unlikely that the nymphs became contaminated by such means, as the sugarcane tissue surrounding the eggs had become quite senescent by the time the nymphs emerged, and the simplest interpretation of this result is that the nymphs acquired FDV from their parents. Regardless of these qualifications, we have demonstrated a sufficiently sensitive means to screen populations of planthoppers at all stages of development for FDV.

7.7 Development of TaqMan assays for SCMV

SCMV is a potyvirus with a linear, non-segmented ssRNA genome. Two TaqMan assays were designed targeting different regions of the SCMV genome. The first assay was designed to detect the highly conserved replicase (NIb) coding region of SCMV. Sequences from eight Australian isolates (Handley *et al.,* 1996) were examined and primer/probe combinations capable of detecting all eight isolates were designed. The second assay was designed to detect the coat protein (CP) coding region of SCMV based on sequences from twelve isolates from Australia, USA and South Africa.

Initial experiments showed that the CP assay was the more robust of the two, possibly due to secondary structure in the NIb region of the SCMV genome causing problems with the reverse transcription reaction. Due to time constraints, it was decided to follow through development of the CP TaqMan assay and to leave further development of the NIb SCMV TaqMan assay to a later date.

An important consideration when optimising and validating the TaqMan assay for SCMV was to select an appropriate template to be used as a standard for quantitation. A ssRNA template is required to act as a control for both the reverse transcription and amplification steps of RT-PCR. However, the purification of intact SCMV ssRNA genomes from sugarcane or maize (i.e. without partial RNA degradation), and uncontaminated by other RNA species, is not practicable.

As a result, it was decided to produce single-stranded RNA transcripts representing the CP region of SCMV using an *in vitro* RNA synthesis system. The following sections describe the production and use of these transcripts for validating the TaqMan assay for SCMV.

7.7.1 Synthesis of *in vitro* RNA transcripts for quantification of SCMV

A 1300bp fragment of the SCMV genome, consisting of the CP coding region and the 3' non-translated coding region, was amplified from a plasmid (pUSN) containing 3'terminus SCMV sequence of an Australian isolate of SCMV using primers CP1 and CPend (all kindly supplied by Laurelea Pickering, BSES, Indooroopilly).

This product was cloned into the vector pCR-SCRIPT which contains both T3 and T7 promoter sequences for the production of RNA transcripts. Resultant clones were characterised and one

clone, pSCR-SCMV.36 was found to contain the correct insert. Using the procedures outlined in Appendix 3, *in vitro* RNA transcripts were synthesised using T7 RNA polymerase. These transcripts were analysed on a formaldehyde agarose gel to check size and quantified using spectrophotometry. The RT-TaqMan assay for SCMV uses the "TaqMan Gold RT-PCR" kit supplied by Applied Biosystems (Appendix 4). Assays are set up in a single PCR tube that contains both Multiscribe reverse transcriptase and Taq Gold DNA polymerase in a single reaction mixture. The reverse transcriptase reaction is completed first, followed by the PCR cycles without openig the reaction tube. The ssRNA SCMV transcripts (produced *in vitro*) were used to prepare a 10 x dilution series ranging from $1x10^7$ to 1 transcript per reaction, and used determine the sensitivity of the assay. The SCMV assay was shown to be quantitative over 5 orders of magnitude, and could reproducibly detect down to 1000 templates per reaction (results not shown). A similar level of sensitivity has been shown for TaqMan detection of tomato spotted wilt virus (TSWV; Roberts *et al.,* 2000), which also possesses a ssRNA genome.

7.7.2 Application of the TaqMan assay for SCMV to field & quarantine samples

An opportunity to test the SCMV TaqMan assay on quarantine material was provided when two international sugarcane leaf tissue samples, showing symptoms similar to SCMV, were available for testing. These samples had been collected by AQIS off-shore and we were requested to screen them using real time PCR. For comparison, a positive control of authentic SCMV-infected sugarcane material, and a negative control grown under FDV-free conditions and presumed to be SCMV-free, were available in our deep freeze (records of source were incomplete).

RNA was extracted from the two quarantine leaf samples, and from leaf and pith tissue of the SCMV-infected and putatively SCMV-free sugarcane, using the FastRNA™ Kit – Green system (supplied by Bio101). Titres were quantified against a 10x serial dilution of SCMV RNA transcripts ($1x10^7$ to 1 copy, quantified by spectrophotometry) as standards.

SCMV was not detected by TaqMan in either of the quarantine samples, a result substantiated by electron microscopy and ELISA tests for SCMV conducted concurrently by QDPI. The veracity of the TaqMan assay procedure was validated by the positive control, i.e. SCMV was detected at high titres in both the leaf and pith tissue of the known SCMV-infected cane (Figure 7.13).

Unexpectedly, TaqMan also detected high titres of SCMV in both pith and leaves of the negative control (presumed to be SCMV-free). Extraction controls and PCR controls were both negative indicating these results were real and that the putative SCMV-free cane was, apparently, SCMV-infected. This sample was certainly asymptomatic for FDV, and may have been asymptomatic for SCMV but phenotypic evidence of SCMV infection would not have been evaluated critically at the time of collection. Because the above assays were done in March 2000 when Project UQ024 was nearing completion and we were fully committed to finalising other priorities, we did not further investigate the source of the SCMV in the putatively SCMV-free leaf and pith samples.

These results clearly illustrate the potential of the SCMV TaqMan assay for field and quarantine testing. Ability to detect SCMV in both leaf and pith tissue is useful, particularly for quarantine samples where often only internodal pith tissue is available. Further work encompassing extended validation of the assay for asymptomatic cane, alternative reverse transcription systems and improving sensitivity levels is required, however, time did not permit these studies to be completed.

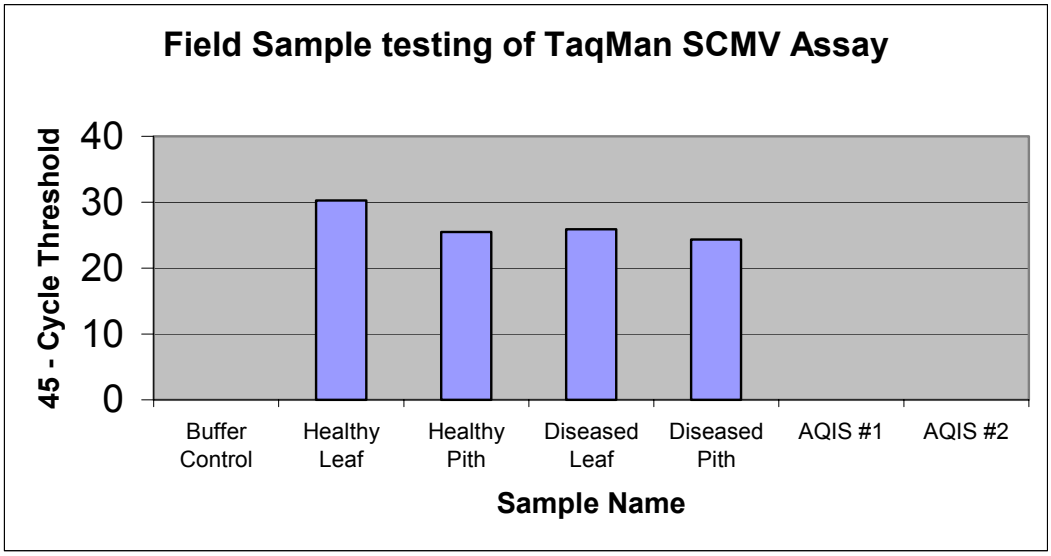

Figure 7.15 Detection of SCMV in field and quarantine samples using the TaqMan assay. Putatively SCMV-free sugarcane was found to contain high titres of SCMV. This result illustrates the ability of the TaqMan test to detect SCMV in asymptomatic cane and in both leaf and pith tissue. Note that extraction buffer control and both AQIS quarantine sample canes were found to be negative for SCMV.

8.0 LIKELY IMPACT FOR AUSTRALIAN SUGAR INDUSTRY

Data presented in this Report show that real time PCR assays such as TaqMan have a number of advantages over alternative approaches to the detection of pathogen-specific nucleic acid targets. As detailed in the next Section, these advantages include enhanced specificity and sensitivity, faster detection, ready adaptability to scale-up and automation, quantitation, and excellent quality assurance capability (which also assists trouble-shooting technical problems). In the longer term, the convenience of obtaining readily validated data in "real time", which avoids the extremely time-consuming and technically-demanding manipulations necessary for alternative nucleic acid-based tests to achieve equivalent specificity and sensitivity (such as PCR-ELISA or procedures requiring gel blots and membrane hybridisation), is expected to provide the greatest benefit.

Therefore, real time PCR technology such as TaqMan has the potential to assist the screening and identification of sugarcane pathogens for a variety of diagnostic applications requiring a molecular test with improved efficiency and reliability.

The experience we have gained using *Cxx*, FDV and SCMV as test pathogens has indicated that TaqMan assays can be developed rapidly for any pathogen for which nucleic acid sequence exists, and that assays for a variety of pathogens can be done under generic conditions. Such efficiencies have the potential to save labour costs for routine pathogen screening, and to enable the sugar industry to respond to emergencies faster and more effectively.

Novel technologies that promise greatly improved performance over existing methods are often treated with caution. Experience is essential to provide a "comfort zone" to potential users. **Therefore, larger-scale uptake of this technology is contingent on ongoing assessment and validation of the TaqMan assays for *Cxx*, FDV and SCMV by BSES**. Only by testing TaqMan with "real world" samples over a period of time, and comparison against alternative tests such as immunological assays and extended plant growth in the glasshouse to allow the development of clear disease symptoms, can the extent of the impact of this technology be gauged. If sufficient support to adopt this ongoing assessment strategy is provided, the capability to deploy this technology quickly will be maintained if a crisis emerges where the attributes of this technology would be of advantage.

9.0 COSTS, POTENTIAL BENEFITS, AND FUTURE RESEARCH NEEDS

9.1 Consumables and operating costs for TaqMan

TaqMan was designed to use 50 µL reaction volumes. We have found that 25 µL reactions work reliably with considerable savings and use this reaction volume routinely. A summary of current costs for TaqMan reagents etc. is presented below for 25 µL reactions (prices are ex GST):

DNA pathogens:

Supplier/item (Current AB price catalogue dated May 2001)	List price	Price per reaction tube
Sigma-Genosys:		
PCR primers, 50 nmole (enables 5,000 x 25 µL reactions)	$100	0.02
Applied Biosystems (AB):		
#450025: TaqMan probe, 5-6 nmole (enables 1,000 x 25 µL reactions)	$540	0.54
#4304437: Universal PCR Master Mix (enables 400 x 25 µL reactions)	$860	2.15
#N801-0933: MicroAmp Optical Tube (pack of 2000)	$440	0.22
#N801-0935: MicroAmp Optical Cap (pack of 2400)	$190	0.08
Use of UQ Real Time PCR Facility must be added to the above costs of consumables:		
Each run on Model 7700 Sequence Detector (96 wells or part thereof)	$50	>$0.52
Minimum cost of reagents/consumables per reaction tube for a DNA template:		**$3.51**

Note that the Universal PCR Master Mix contains nucleoside triphosphates, passive reference dye (ROX - essential for accurate quantitative data), *Taq* DNA polymerase, and AmpErase/UNG (enzyme for degradation of previous PCR products synthesised using dUTP in place of dTTP).

RNA pathogens:

RNA viruses require a reverse transcriptase step prior to TaqMan PCR detection. Generally this is done in two separate steps, the first being a reverse transcription reaction in one tube; a sample of the reaction is transferred to a second tube for the TaqMan step. We have used two complete kits from Applied Biosystems to develop the FDV and SCMV assays. These kits allow the two steps to be completed sequentially in a single reaction tube, using different temperature programs in the Model 7700 Sequence Detector without opening the tube:

Supplier/item (Current AB price catalogue dated May 2001)	List price	Price per reaction tube
for SCMV RT-TaqMan assay		
#N808-0232: (uses *Taq* DNA polymerase + multiscribe RT enzyme)		
TaqMan Gold RT-PCR Core Reagents (for 400 x 25 µL reactions)	$1,540	$3.85
for FDV RT-TaqMan assay		
#N808-0232: (uses *Tth* DNA polymerase for both RT and PCR steps)		
TaqMan EZ RT-PCR Core Reagents (for 400 x 25 µL reactions)	$1,540	"
Additional costs:		
PCR primers, 50 nmole (enables 5,000 x 25 µL reactions)	$100	0.02
#450025: TaqMan probe, 5-6 nmole (enables 1,000 x 25 µL reactions)	$540	0.54
#N801-0933: MicroAmp Optical Tube (pack of 2000)	$440	0.22
#N801-0935: MicroAmp Optical Cap (pack of 2400)	$190	0.08
Each run on Model 7700 Sequence Detector (96 wells or part thereof)	$50	>$0.52
Minimum cost of reagents/consumables per reaction tube for an RNA template:		**$5.23**

9.2 Cost comparison of TaqMan with alternative diagnostic technologies

EB-EIA assay for Cxx (detection of RSD)
The cost per reaction for detection of RSD by EB-EIA is about $0.30 for reagents and $0.70 for labour (based on cost recovery estimates by B. Croft, BSES). This can be compared to the above estimate of $3.51 per reaction for the TaqMan assay for RSD (detection of *Cxx*), without attempting to estimate labour costs. If equipment capital costs and maintenance are not an issue, the cost of consumables per reaction for the TaqMan assay would drop to ~$3.00 per reaction. Thus the cost of consumables for TaqMan ($3-3.50) are not competitive with EB-EIA ($0.30) at present, and unlikely in the future.

PCR-ELISA for Cxx (detection of RSD)
In 1998 when we were comparing TaqMan and PCR-ELISA as a generic diagnostic platform for further method development, we made a price comparison. The cost of immunologicals and other consumables for PCR-ELISA (based on kits supplied by Boehringer before takeover by Roche) was appreciably greater than costs for TaqMan. Using list prices at the time, e.g. for 25 µL reaction volumes, costs per single reaction were:

	DNA pathogens	RNA pathogens
PCR-ELISA	$5.19	$8.72
TaqMan	$2.61	$4.02

Possibilities for cost savings:
We have used quality assured reagents and kits supplied by Applied Biosystems to develop, optimise and validate the TaqMan assays for *Cxx*, FDV and SCMV. This approach establishes a secure benchmark for future use. Cost savings are possible by sourcing alternative PCR tubes and preparing our own reagents instead of using kits. The ROX passive reference is not available individually and could be omitted if accurate quantification is not required. The reverse transcriptase kits are particularly expensive, and we have shown that considerable savings can be made by purchasing reverse transcriptase separately and adding it to standard TaqMan assay reagents. However further research would be necessary to optimise and validate these cost-saving strategies. This would be justified if large-scale pathogen screening programs were envisaged.

Equipment costs for TaqMan
Minimum PCR thermal cyclers capable of running TaqMan assays are in the vicinity of $100K, although costs are expected to drop as real time PCR becomes an established technology. Current costs are therefore somewhat greater than the ELISA plate readers etc. used for EB-EIA. Robotic workstations for automation of PCR reaction setups are currently under development with an approximate price in the range $30-50K (estimate based on confidential discussions of DJ Maclean with industry sources), and would considerably decrease labour time and human error for TaqMan analysis.

9.3 Generic benefits of TaqMan for pathogen diagnostics

The inherent and demonstrated benefits of TaqMan assays compared to gel electrophoresis and PCR-ELISA for detecting sugarcane pathogens are listed below. ***These benefits can best be summarised as improved assay streamlining, quantitation, and quality control as detailed below:***

(a) Generic assay design parameters save considerable time in developing assays for new pathogens.

(b) Pathogen detection is in "real" PCR time i.e. no post-PCR manipulation other than computer analysis, and data can be downloaded or transferred over the internet.

(c) Less possibility of cross-contamination by aerosols etc. generated by previous assays, should give rise to fewer false positives generated by sample contamination.

(d) The kinetics of PCR amplification in real time validates a positive result even at low titre, compared to the doubt engendered by a faint band in a gel or a faint colour in an ELISA plate. This minimises the possibility of false positives arising from non-specific "signal noise" that is always likely with end-point detection methods.

(e) Patterns given by raw spectral data accumulated during the PCR run can be used to validate the computer algorithms used to generate amplification plots, and to troubleshoot the cause of poor amplification patterns if they arise. This adds an extra element to quality assurance by eliminating another source of false positives.

(f) As with PCR-ELISA, internal hybridisation probes provide an extra level of specificity and minimise the possibility of false positives compared to gel-based detection.

(g) The process is quantitative over a wide dynamic range of six or more orders of magnitude.

(h) Low-titre samples can be assayed routinely and reliably, without compromising quantitative data from the occasional high titre sample and without re-assaying.

(i) Two different nucleic acid targets can be multiplexed (assayed simultaneously) in the same tube, providing the potential to develop internal controls to identify false negatives due to inhibitors finding their way into a sample of if an error occurs during reaction set-up.

(j) Multiplexing can also enable two different pathogens to be assayed in the same tube.

(k) The potential exists to set up long-lived kits for specific pathogens with dry primer/probe in reaction tubes, requiring addition only of sample and a general Universal master reagent.

(l) Generic assay set-up: all assays are designed to use the same reaction conditions, enabling different reactions for different purposes to be assayed in the same run.

(m) Items (g) - (l) should enable small-scale tests that are uneconomic individually to be run along with other tests in a routine diagnostic laboratory with minimal specialist input.

(n) The TaqMan assay appears to be more robust in the presence of inhibitors of PCR due to its ability to detect product amplification at low threshold levels.

(o) The technology has the potential to be extended to automated germplasm screening for specific molecular markers in breeding programs.

(p) The technology can be used for quantitative gene expression analysis in breeding programs and various research applications.

9.4 Limitations and alternatives to TaqMan for pathogen diagnostics

Major limitations to the adoption of TaqMan technology are perceptions of high costs and lack of familarity with the technology. Furthermore, highly sensitive analytical systems such as TaqMan can more easily detect false positives due to sample contamination, and doubt generated for whatever reason causes problems of acceptability. Issues related to this high sensitivity are discussed in detail in Section 5.2, subsection 5 (ii) and Appendix 5 of this report. Other limitations include:

(a) Equipment for TaqMan is expensive and is currently unaffordable by many routine diagnostic laboratories. This equipment is readily accessible to BSES through the

UQ Real Time PCR Facility (RTPF). In the longer term we believe that equipment will improve in performance and reduce in price as real time PCR is taken up more widely via competition among suppliers. Currently, the Corbett Rotor-Gene is much less expensive but has far fewer features than the Applied Biosystems ABI-Prism model 7700 Sequence Detector that was used in this study. We do not believe that any of the alternative real time PCR systems currently available from other suppliers (e.g. Roche, Stratagene, BioRad, Corbett) have any technical advantages over the Applied Biosystems Model 7700 Sequence Detector which we believe to be the most versatile instrument. However, these alternative instruments can also run TaqMan assays. Applied Biosystems also make cheaper instruments that the Model 7700 Sequence Detector, as well as more expensive high-throughput (384 well) instruments.

(b) Consumables for TaqMan are expensive, but are comparable to PCR-ELISA and alternative detection technologies that provide the extra level of specificity of an internal hybridisation probe (e.g. Southern blotting after gel electrophoresis). Alternative hybridisation probes to TaqMan (e.g. Molecular Beacons) may be advantageous in some circumstances, but this will require research to evaluate, and the TaqMan probes we have used to date work very well.

(c) Alternative "real time" technologies to TaqMan PCR. Alternatives to PCR that use isothermal reaction conditions to amplify signals from specific nucleic acid sequences are in the process of becoming commercially available e.g. "NASBA" (developed by Organon) and "Invader" (Third Wave Technologies). These technologies can be adapted to real time signal detection. Research and experience will be necessary to evaluate the advantages and disadvantages of these systems compared to real time PCR. **However, they will be subject to protected IP and are unlikely to provide great cost advantages to TaqMan, and will be subject to the same general advantages and limitations of nucleic acid-based diagnostics as TaqMan.** These issues are expected to be sorted out by experience in the market place over the next decade.

(d) Novel end-point nucleic acid technologies are also becoming available e.g. "First Change" developed by QUT. Similarly to the Invader technology described above, these alternative end-point technologies have been designed primarily to detect single nucleotide substitutions for use as genetic markers e.g. for detection of genetic disease or for genetic selection during breeding, and appear to be less amenable than TaqMan for pathogen diagnostics.

The above issues are expected to be sorted out by experience and the market place over the next decade as these new technologies are compared to each other and to alternative technologies based on immunology etc. It is expected that experience with TaqMan will facilitate the uptake of alternative nucleic acid-based technologies if it becomes desirable. Increased costs are expected to be counterbalanced by increases in efficiency with real time detection technologies.

9.5 Potential benefits of the TaqMan assay for Cxx

Ratoon stunting disease is currently managed by testing canes later in the growth cycle when pathogen titres generally reach levels readily detectable with the existing EB-EIA. Hence there is no urgent need for large-scale screening with the TaqMan assay (indeed, *Cxx* was selected as initial model to assess TaqMan because the existing EB-EIA provided such a good benchmark). There are some limitations with the existing EB-EIA however, including (1) it is necessary to collect and individually test xylem fibrovascular fluid (FVF) obtained from each of many stalks sampled from each field, which is very time-consuming, (2) for some potential applications (e.g.

young canes) *Cxx* titres are likely to be below the detection limit of EB-EIA, (3) EB-EIA cannot distinguish *Cxx* from *Clavibacter xyli* subsp. *cynodontis* (*Cxc*). Potential uses for the TaqMan assay therefore include:

(a) Retesting to confirm doubtful EB-EIA results for important field or quarantine samples.

(b) Analysis of young field or quarantine canes when *Cxx* titres are likely to be low and xylem fibrovascular fluids (FVF) are difficult to obtain or in low quantity.

(c) Testing of pooled samples, e.g. from FVF or pith collected from many samples in the one field, if infected canes are likely to be infrequent or scattered across the field. Such a mixed sample will dilute the *Cxx* titre, and will require the extra sensitivity of TaqMan to detect the pathogen reliably. This strategy could have commercial applications as testing of one pooled sample per field would be logistically simpler and more cost-effective.

(d) In situations where unexplained outbreaks of RSD occur, the TaqMan assay could be adapted to large-scale screening of adjacent grasses to determine if *Cxx* is harbored in addition to (or in place of) *Cxc*.

(e) Multiplexed quarantine indexing (see 10.7 below)

9.6 Potential benefits of the TaqMan assay for FDV

FDV is currently managed by breeding for resistance, and by limiting the movement of potentially diseased planting material because the leafhopper vector *Perkinsiella saccharicida* is not thought to move over large distances. As discussed above, PCR-based assays can detect FDV in the internodal pith of known infected cane, in leafhoppers at various stages of development, and in the nymphs of eggs freshly hatched from FDV-infected cane. Potential uses of the TaqMan assay include:

(a) Testing for FDV in crops or resident populations of the insect vector, to detect the presence of FDV before the appearance of visible disease symptoms.

(b) Tracking the movement of the insect vector and its ability to maintain and transmit FDV.

(c) Multiplexed quarantine indexing (see 10.7 below)

9.7 Potential benefits of the TaqMan assay for SCMV

SCMV is currently managed by deployment of varieties that show resistance to the SCMV strains currently present in Australia, but outbreaks and disease loss can occur with some varieties. Disease is spread by various insect vectors. The greatest threat comes from genetically divergent strains currently absent from Australia. Potential uses of the TaqMan assay include:

(a) Testing for SCMV in crops or resident populations of the insect vectors, to detect the virus before the appearance of visible disease symptoms.

(b) Tracking the movement of insect vectors and their ability to maintain and transmit virus.

(c) Testing overseas accessions of sugarcane and other grasses known to be infected with mosaic virus, to determine the spectrum of strains that can be detected with the current test or modified tests containing redesigned PCR primers and probes.

(d) Multiplexed quarantine indexing (see 10.7 below)

9.8 Future research & development opportunities for TaqMan technology

The pioneering work on TaqMan trialled with *Cxx*, FDV and SCMV in this project has enabled us to assess the suitability of this technology for a number of applications of interest to the sugar industry. For many of these applications, we believe that real time PCR is the best (or only feasible) approach. Such applications include the following:

(a) Multiplexed TaqMan assays for indexing pathogens in sugarcane germplasm

- e.g. using total nucleic acids extracted directly from pith of imported setts or from biopsies of young canes growing from the setts. The same nucleic acid extract could be assayed for a variety of pathogens by PCR. TaqMan assay protocols are developed in a common format suitable for the simultaneous analysis of a variety of different targets.
- SRDC Project BSS187 developed suitable methods of nucleic acid extraction from pith tissue of quarantine material prior to planting. These methods can be extended to glasshouse and field canes.

(b) Development of quantitative TaqMan assays for additional pathogens. Examples include:

- Viral and phytoplasma agents associated with Yellow Leaf Syndrome, to explore relationships between absolute and relative titres of these two agents that give rise to disease phenotypes.
- *Xanthomonas albilineans* causing leaf scald disease, e.g. to screen exotic germplasm (some overseas strains show differing virulence patterns to known Australian strains), or to evaluate germplasm resistance in breeding programs.

(c) Evaluation of disease resistance using TaqMan assays (including disease etiology)

- Quantitative analysis of pathogen titres after inoculation of breeding populations, to select lines showing greatest resistance to pathogen buildup.
- This approach is most applicable to bacterial pathogens (e.g. *Xanthomonas albilineans*) or viral pathogens (e.g. FDV) where there is an industry need for such analysis, and alternative methods are either equivocal or time-consuming.
- Quantitative assays can be used to track the build up and movement of a pathogen in different parts of the plant during disease development.

(d) Pathogenesis gene expression using TaqMan assays for transcript abundance

- The same approach used to develop RT-PCR TaqMan assays for SCMV can be used to develop quantitative TaqMan assays for mRNA levels of pathogenesis genes during growth of the pathogen in the plant. DJ Maclean's laboratory has demonstrated the utility of this approach for expression of a pathogenicity gene during infection of the forage legume *Stylosanthes* by the fungal pathogen *Colletotrichum gloeosporioides*, and similar assays could be developed for putative pathogenicity genes of sugarcane pathogens. Given that many pathogens such as

Cxx grow thinly within host tissues, it is difficult to do such expression analysis of pathogenesis genes in any other way.

(e) Sugarcane gene and transgene expression using TaqMan assays for transcript abundance

- Using a similar approach to the expression of pathogenesis genes, TaqMan assays could be developed to assess the levels of expression of sugarcane genes of interest (e.g. genes associated with induced resistance, or enzymes required for sucrose synthesis and accumulation) in different parts of the plant and in different varieties.
- Gene discovery projects using microarrays have identified expressed sequence tags (ESTs) in sugarcane as part of SRDC Project CTA035. Collaboratively with CTA035, expression levels of selected ESTs of interest have been assayed by an adaptation of real time PCR in our laboratory.

(f) Improvement to protocols for diagnostic TaqMan assays.

The generic assay protocols we have developed for *Cxx*, FDV and SCMV are capable of further optimisation requiring further research. This includes:

- Development of cheaper in-house reagents for TaqMan PCR compared to kits supplied by Applied Biosystems.
- Development of long-lived on-the-shelf kits for pathogens of interest which could be prepared in-house. For example, primer/probe combinations for specific assays could be dried in assay tubes and stored in the freezer. Assay mixtures could simply be prepared by adding sample, Universal assay reagent, and water to volume. Such kits could enable a rapid response to a crisis, or efficient and labor-effective assays for various pathogens at the same time, on a routine basis.
- Blocking agents and treatment of samples require further exploration, to minimise further the possibility that inhibitors of Taq polymerase could give false negatives. Such inhibitors are generally removed by sample processing etc. but have the potential to find their way into some samples.
- **Development of a multiplexed internal quality control**, i.e. a TaqMan assay for a sugarcane "housekeeping gene" that can be included in the same reaction tube as an assay for a pathogen . If an inhibitor of Taq polymerase is present in a tissue extract of sugarcane, the null result for the internal control will indicate that the test for the pathogen is null and void. Such samples would require reworking and retesting. Development of such a multiplexed internal control was included in Milestone 8 as part of the Review of UQ024 held in 1998, but was not achieved due to prioritisation of further field tests using the *Cxx* assay before funding of UQ024 ceased on 30/6/2000.

10.0 COMMERCIAL SIGNIFICANCE OF PROJECT TECHNOLOGY

10.1 IP and licencing issues

1. Legal considerations

The views presented below represent the current opinion of the writers of this Report and are not binding. Any commercial users of PCR and TaqMan technology should seek independent legal advice on the issues discussed below.

The assays developed in this project used nucleotide sequence information largely obtained in other projects. Commercial applications of these assays will be subject to the PCR patent, managed for agricultural applications by Applied Biosystems for Roche Pharmaceuticals. A number of alternative chemistries are available for the real time detection of PCR products (and more are likely to emerge including alternatives to PCR for signal amplification from a specific nucleic acid sequence), all of which are expected to be protected by various patents. At the time of writing this report we believe the TaqMan™ system commercialised by Applied Biosystems is the most robust real time PCR system available, it is well supported by Applied Biosystems, and it will be simpler (and probably of advantage) to deal with a single licencing authority (Applied Biosystems) for all aspects of PCR technology including real time detection of PCR products.

Licencing costs for commercial applications of PCR itself (without any additional IP-protected sample processing or detection technologies) are currently a one-off licence fee of US$10K + 15% of the charge to customers for conducting each test, with the 15% charges in the first year deducted from the US$10K licence fee. Unofficially, we have been advised by a local representative of Applied Biosystems that they may add a small extra charge for the use of TaqMan, say 5%, to the 15% charge applicable to the PCR patent. **It is emphasised that <u>any</u> commercial application of PCR, including gel electrophoresis-based detection of PCR products, will need to be licenced through Applied Biosystems or their nominee (e.g. another holder of a licence within Australia). The <u>above statements</u> have been checked with Applied Biosystems for accuracy. Presumably, use of TaqMan will incur no charge until Applied Biosystems define their position.**

The Institute for Horticultural Development (IHD) at Knoxville, Agriculture Victoria, has purchased a PCR licence fee that can cover all commercial phytopathogen testing in Australia. However, as we understand it, this licence was negotiated with Applied Biosystems to cover 15% of the total cost of the test including sample processing and advice, and it must be questioned whether this arrangement will suit the needs of the sugar industry where bulk testing is done, and commercial applications are not clearly separated from research. We presume PCR licences have also been obtained (or will be obtained) by SARDI (South Australian Research & Development Institute), Aventis, and other organisations within Australia for phytopathogen diagnostics. Applied Biosystems will deem a 15% licence charge if they think the commercial value of a test is too low. These issues can be negotiated.

At some stage in the future the sugar industry will have to adopt a position on licencing arrangements for use of the PCR patent and downstream detection technologies. However, we believe that while new TaqMan assays for particular pathogens are under development and validation, they constitute "research" and not "commercial" applications of the IP-protected technologies. As long as this situation can be sustained, and while the tests under validation are being done in licenced thermal cyclers using licenced thermostable DNA polymerase, it can be argued that the tests will not be liable for the payment of commercial royalty charges. In the longer term, two possible courses of action are suggested:

(a) After each PCR-based assay has been developed and validated, its commercial application can be contracted out e.g. to IHD, SARDI, Aventis, or an agency set up by BSES. In such a scenario, TaqMan assays conducted in-house by BSES for quarantine indexing may escape the PCR and TaqMan licencing fees provided it can be demonstrated that such tests are purely research. This argument may be difficult to sustain, for example, if it can be counter-argued that quarantine indexing will lead to the creation and release of new cultivars of commercial value.

(b) The sugar industry can negotiate its own industry-wide licence for the use of PCR (and TaqMan when Applied Biosystems have defined their position) that will cover bulk pathogen testing as well as PCR-based marker technology that can be used for the development of new sugarcane cultivars.

The above comments have not been checked by any legal authority and have been formulated to raise issues and options for discussion. It may be advantageous for a research body such as SRDC, rather than BSES which also implements plant protection and improvement programs, to negotiate with Applied Biosystems for an industry-wide licence for use of the above patents, in order to increase the perception that most applications are research-based rather than commercial.

1. Some practical considerations

Real time technologies of any sort are unlikely to compete for reagent costs with simple gel-based detection (although time savings and improved reliability may counterbalance cheaper consumables). However if royalties for use of PCR/TaqMan patents are liable for fee-for-service commercial applications, the cost of the licence fee is small compared to the overall charge for the service when that the test outperforms alternative detection technologies. This point was made by Jane Moran during the review of UQ024 held in September 1998, based on her experience in running a commercial diagnostic service through IHD. In such situations, the extra cost of consumables for a reliable test would be small compared to the total cost of the service.

10.2 IP protection of assay systems developed for specific sugarcane pathogens

As noted above, the assays developed in this project used nucleotide sequence information largely obtained in other projects (the one possible exception [as part of Honours research of L. Pickering] was sequencing of target FDV sequences from various Australian isolates to identify polymorphisms present in field populations, and to identify apparently invariant sequences for probe and primer design). We understand it is possible to patent the TaqMan assay for each pathogen based on the specific PCR primers and TaqMan probes and any unique aspects of the assay procedure (such patents would be added to the PCR and TaqMan patents). However, unless the patenting costs are likely to be recovered by charging licencing fees to testing laboratories charging commercial fees for the tests, or unless the strategic interests of the Australian sugar industry are best protected by denying the tests to overseas competitors via patent protection, we would advise against developing patents. Protection can also be obtained by trade secrecy or disclosure such as by publication. Each of the specific TaqMan assays developed during this project is considered below by the above criteria:

Cxx assay

Cxx is currently managed by a robust EB-EIA assay that is not patented or patentable. The greater sensitivity and quality assurance inherent in the TaqMan assay for *Cxx* would be of advantage for the following potential commercial applications, none of which are believed to be likely to generate sufficient profits to recoup patenting costs:

(a) Retesting/confirmation of doubtful samples previously assessed by EB-EIA or phase contrast microscopy.

(b) Bulk testing of pooled field samples either (i) to detect the presence of RSD in a crop, or (ii) to verify the RSD-free status of certified seed cane.

(c) Quarantine indexing of germplasm (overseas accessions and breeding populations) that may lead to the production and sale of new varieties.

FDV assay

FDV is currently managed by deployment of resistant varieties, and by monitoring or restricting the movement of potentially diseased cane. The TaqMan assay for FDV has the following potential uses, none of which are believed to be likely to generate sufficient profits to recoup patenting costs:

(a) Testing for the presence of FDV in crops before the appearance of visible symptoms.

(b) Testing for the presence of FDV in the insect vector of FDV as a marker for the presence of FDV in the field and to develop better strategies to suppress or avoid insect-mediated transmission.

(c) Quarantine indexing of germplasm (overseas accessions and breeding populations) that may lead to the production and sale of new varieties.

SCMV assay

SCMV is currently managed by deployment of varieties that are resistant to the SCMV strains currently present in Australia. The greatest threat comes from genetically divergent strains currently absent from Australia, and further research will be necessary to evaluate whether a PCR-based assay such as TaqMan will be able to detect all members of this virus group. The TaqMan assay for SCMV has the following potential uses, none of which are believed to be likely to generate sufficient profits to recoup patenting costs:

(a) Testing for the presence of SCMV in crops before the appearance of visible symptoms.

(b) Quarantine indexing of germplasm (overseas accessions and breeding populations) that may lead to the production and sale of new varieties.

11.0 OTHER TECHNICAL INFORMATION DEVELOPED DURING PROJECT

nil (all has been described in Sections 7.0, 8.0 and the Appendices)

12.0 RECOMMENDATIONS

This Report presents evidence that the TaqMan real time PCR assays developed in Project UQ024 have many potential advantages over current approaches for the efficient and reliable screening of sugarcane germplasm to detect known pathogens. Novel technologies that promise greatly improved performance over existing methods are often treated with caution. Accordingly:

1. **It is recommended that the sugar industry (e.g. SRDC) assists BSES to support the ongoing implementation and validation of the TaqMan assays developed to date: *Clavibacter xyli* subsp. *xyli* (*Cxx*) causing Ratoon Stunting Disease, Fiji Disease Fijivirus (FDV), and Sugarcane Mosaic Virus (SCMV)**.

Support for ongoing validation of the above assays will also maintain the capability to deploy this technology quickly if a crisis emerges where its attributes would be of advantage. In the longer term, the validation process will provide the necessary experience to judge when this

technology should be deployed more widely to assist various ongoing needs and research oportunities for the sugar industry.

Quality assurance is a key issue when screening breeding germplasm for disease. The digression of Project UQ024 to assess the cause of false positives revealed by the TaqMan *Cxx* assay resulted in other milestones not being completed. Therefore:

2. **It is recommended that SRDC fund the development of a multiplexed TaqMan quality control system to enable the detection of false negatives in diagnostic assays for sugarcane pathogens.**

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Appendix 1

Issues in quality control for PCR-based diagnostic assays of sugarcane pathogens

Issues in quality control:
(**Adapted from Attachment 5 from Milestone 8 Report of SRDC Project UQ024)**

DEVELOPMENT OF QUALITY CONTROL PROCEDURES FOR PCR-BASED DIAGNOSTIC ASSAYS OF SUGARCANE PATHOGENS

Ideally, diagnostic technologies require development of the following quality control procedures:

(1) Detection and avoidance of false positives (e.g. due to contamination of pathogen-free samples with exogenous pathogen cells, or PCR products from previous assays)
(2) Detection and avoidance of false negatives (e.g. due to the presence of inhibitors of PCR that prevent the amplification of DNA from samples that do contain intact nucleic acid target sequences of the pathogen).
(3) Assessment of inaccuracy and imprecision of quantitative data (i.e. how close are estimated values to actual titres within the tissue samples, and what errors are generated in repetitive assays to determine such values?). In situations where accurate estimates of pathogen titres within tissues are required, it is necessary to estimate recovery efficiencies of pathogen target sequences after tissue extraction and assay.

For sugarcane quarantine indexing, quality control procedures (1) and (2) are of vital importance to prevent: (a) the unnecessary destruction of valuable breeding germplasm (due to false positives), and (b) to prevent "escapes" of an exotic pathogen into breeding populations and the field by failure to detect it in the original germplasm accession due to false negatives. Procedure (3) is less of an issue for quarantine indexing as the presence of any viable propagules of a pathogen in germplasm have the potential to lead to an escape. However, quantitative data on the limits of detection, and relative titres in the various tissues sampled for assay, have an important bearing on the type of routine assay procedures developed and their interpretation. Furthermore, assays developed for quarantine indexing can be adapted to other purposes such as pathogen detection in field samples for routine disease management, and for assessment of germplasm resistance by evaluation of pathogen levels in inoculated tissues.

With the development of real time PCR detection technologies such as TaqMan, it is now possible to develop routine, ultra-sensitive assays capable of detecting <10 specific target DNA or RNA sequences with high sensitivity, and to obtain quantitative data on a routine basis with little effort other than the actual PCR assay itself. Although the high specificity of TaqMan makes it less likely that false positives would be detected by cross-reactivity with related pathogens (as for immunological-based diagnostics), the high sensitivity of TaqMan makes it highly likely that false positives might be detected by cross-contamination, either by "stray" cellular material or propagules of the pathogen in the environment, or by aerosols containing PCR products from previous assays. ***The origin and consequences of false positives and false negatives are considered in depth below.***

1. False positives.

Two major causes of false positives are envisaged:

(a) ***Laboratory contamination:*** e.g. aerosols generated from previous PCR assays, or preparations of positive controls such as recombinant plasmids containing the cloned target sequence. Such contaminants frequently find their way into the RO water supply in a laboratory, and can be present as particles in the air. Such problems are avoided by a combination of improved laboratory hygiene, use of a "clean room" to set up all components of reaction

mixtures other than sample addition, preparation of stock reagents in the laboratories of colleagues in distant locations (and use of RO water from such sources). Laboratory contamination from previous PCR assays is minimised by TaqMan product detection, as no post-PCR detection steps are necessary. Also, we generally pre-treat reaction mixtures with the enzyme uracil-N-glycosidase (UNG) to degrade possible contaminants originating from previous assays (UNG cannot be used for assays that detect RNA viruses).

(b) ***Environmental contamination:*** air-borne cells or particles of a pathogen, e.g. aerosols and fluids generated during collection of samples from infected material, can contaminate uninfected samples collected at the same location. This problem can be avoided by the development of improved sample collection procedures. In many situations, contaminated samples would be expected to show lower pathogen titres than authentically-infected samples, and experience with a quantitative assay system such as TaqMan is necessary to determine the "normal range" of titres in infected sugarcane. However, a low pathogen titre can be also be obtained from samples at early stages of infection or if the pathogen is unevenly distributed within host tissue, and such samples would generally require extensive retesting to ensure a reliable result, and hence avoid unnecessary destruction of valuable germplasm.

It is sometimes suggested that assay procedures can be "too sensitive" if they are able to detect low levels of cross-contamination. To the contrary, we would argue that provided the assay procedure is highly specific for the pathogen, and that a high background "noise" cannot be confused with a positive result, it is better to know what is actually present in a sample, enabling an informed decision to be made for further action. One of the virtues of real time TaqMan-PCR assays is that a positive result is easily recognised by the kinetics of specific DNA amplification, and can be readily distinguished from background fluorescence signals. As discussed below however, a negative result does not necessarily prove the absence of a pathogen.

How TaqMan Multi-component analysis can detect false positives

As noted above, any analytical system has the potential for some reactions to generate signal noise that can be mistaken for a positive result. The multi-component analysis facility of the Model 7700 Sequence Detector is particularly useful for assessing the validity of a doubtful result from a TaqMan assay. This instrument collects a full fluorescence emission spectrum contributed from dyes activated during PCR plus the quencher dye (usually TAMARA) and the internal reference (ROX). Algorithms in the data analysis software integrate these data to produce an "amplification plot" showing relative fluorescence *vs* cycle number for the specific nucleic acid target sequence being amplified. ***Exponential amplification kinetics indicates that an authentic PCR amplification has occurred, and validates a result from even a single nucleic acid target molecule in a sample.***

On some occasions we have observed irregular amplification plots that appear to be distorted by signal noise in the reaction. To check the veracity of the amplification plot, the raw spectral data accumulated during the assay can be resolved into the output of each component dye present in the reaction mixture (this is the "multi-component" plot). ***The multi-component plot can discriminate reactions where signal noise emanating from impurities in a reaction produces a false or irregular amplification plot compared to an authentic amplification plot.***

The full emission spectrum collected by the Model 7700 Sequence Detector has another virtue in that it can enable TaqMan assays for two different nucleic acid targets to be "multiplexed" in the same reaction (i.e. simultaneous assay in a single tube). This virtue can be used to develop an internal quality control assay to eliminate possible false negatives, as discussed below.

2. False negatives

Three major causes of false negatives are envisaged:

(a) Sample heterogeneity: PCR-based tests are most useful for pathogens that do not cause distinctive disease symptoms. When a pathogen resides in apparently "healthy" cane (e.g. in latent infections, or at early stages of infection before symptoms are evident), it may be unevenly distributed. This potential problem requires research to determine the most appropriate tissues to sample, and may require pooling, homogenisation, and sub-sampling. Highly specific and sensitive assays (e.g. TaqMan) with the potential to detect low pathogen titres will assist the resolution of such problems.

(b) Sample degradation: During the process of preparation of samples for PCR reactions, target DNA (or RNA) template can be degraded by either the extraction procedure, or the conditions of transport and storage of either biological samples or nucleic acid extracts. This potential problem requires research to determine the most appropriate extraction procedures, and transport/storage conditions for particular cane materials and extracts thereof.

(c) Inhibitors of PCR: Whereas items (a) and (b) above apply to any nucleic acid-based diagnostic system (and indeed to many other diagnostic technologies), inhibitors of the polymerase chain reaction (commonly called *Taq* inhibitors) are peculiar to this technology. Although prior research can enable the selection and development of nucleic acid extraction procedures that are generally PCR-competent, some samples may contain sufficient inhibitor to affect the amplification efficiency, and in some situations the reaction may not work at all. Such inhibitors can arise from causes such as inefficient purification of some samples in a batch, or from exogenous materials unwittingly (and intermittently) finding their way into PCR reaction mixtures. Most workers using PCR have experienced this problem. Inhibitors also exist for reverse transcriptase (RT), an enzyme necessary for the assay of RNA viruses by PCR.

As noted above, causes (a) and (b) can be minimised by prior research to design appropriate sample management and processing procedures. Despite such precautions, experience shows that inhibitors of PCR frequently cause false negatives. PCR reactions can be monitored for the presence of such inhibitors by "spiking" the assay mixture with authentic DNA (or RNA) template for either the same sequence target or another sequence (**external positive control**). If the predicted DNA amplification product(s) are not produced, it can be deduced that the sample contains an inhibitor of PCR (or RT-PCR). Alternatively, the sample itself can be spiked with the control target sequence, which is carried through all extraction steps and into the final PCR reaction (**internal positive control**). A third approach can be taken if the sample is expected to contain DNA (or RNA) sequences derived from sources other that the pathogen, e.g. a sugarcane gene; in this case the nucleic acid extract can be also assayed for this other sequence (**endogenous positive control**).

A multiplexed positive control can detect possible false negatives

As a consequence of Review I of Project UQ024 held September 1998, Milestone 8 was revised to include the development of an endogenous positive control to monitor PCR reactions for false negatives. The intended strategy was to interact with SRDC-funded project CTA035 "A sugarcane gene bank" which is identifying expressed sequence tags (ESTs). ESTs represent genes that are expressed as messenger RNA in various sugarcane tissues, and CTA035 is identifying genes that are expressed abundantly in meristematic and mature stem tissue, and in roots challenged by substances that induce pathogen defense genes. Database analysis of these

ESTs can identify a suitable sugarcane "housekeeping" gene that is abundantly expressed in various sugarcane tissues, which can be used to develop a TaqMan assay suitable as an endogenous positive control. ***The great advantage of this strategy is the ability to monitor the positive control IN THE SAME REACTION TUBE as the pathogen assay.***

This strategy is particularly important for the detection of RNA viruses in valuable breeding germplasm. Protocols will include multiplexed assays for both the sugarcane EST and the pathogen gene within the same RT-PCR-TaqMan reaction tube. Because nucleic acid extracts of sugarcane germplasm will contain messenger RNA representing the EST sequence, the multiplexed RT-TaqMan assay will be expected to give a positive assay result for this sequence. Hence, a sample which gives a positive assay result for the sugarcane EST as an endogenous positive control, but a negative result for a pathogen sequence, can be confidently deemed a true negative for the pathogen. ***Conversely, a negative result for the endogenous positive control indicates that the PCR reaction did not work correctly*** (e.g. due to faulty reaction set-up or the presence of Taq or RT inhibitors). ***Thus, if the PCR reaction fails to detect the endogenous control FOR ANY REASON, a negative result for the pathogen assay will immediately be identified as a potential false negative, and the assay result rejected.*** Such quality assurance is a great advantage when assaying valuable germplasm stocks, when a reliable result is essential to ensure that pathogen escapes are avoided.

The above principle can be applied to assays for DNA targets (e.g. bacterial or fungi) where the endogenous positive control is based on the genomic sequence representing the EST.

Appendix 2

Technique for the collection of FVF from sugarcane

Collection of sugarcane fibrovascular fluid (FVF) for RSD assay

Materials Required: Large pair of secateurs, 70% Ethanol spray, positive pressure pump (constructed from a milking machine teat attached to the hose of a car battery operated air pump. A switch is connected close to the open end of the teat attachment to make operation of the pump more convenient), storage rack, screw-capped, O-ring collection tubes, template for record of samples, marker pen.

1. Cut complete stalk of cane to be tested close to the ground
2. Remove leaves for ease of handling
3. Sterilise secateurs with 70% ethanol
4. Cut the cane stalk into pieces such that each section contains one internode and maximum amount of internodal stalk. Cutting one end of the cane piece on a diagonal makes collection into tubes easier
5. Place cane piece in end of teat, hold firmly to seal and press switch to start pump. Collect FVF drops in tubes.
6. Continue with remaining stalk pieces until between 500μL and 1mL of FVF has been collected
7. Place lids on tubes and transport on wet ice. Store at –20^{o}C as soon as possible.

Notes:

1. *Cxx* colonises close to the base of the cane plant. Therefore, the highest concentration of *Cxx* cells will be in pieces cut from closest the roots

2. Most of the FVF is collected during the first few seconds of postive-pressure application. When bubbles begin to appear on the cut surface of the cane, no more FVF is obtainable.

3. Surface sterilize secateurs between cane setts to prevent cross-contamination.

Appendix 3

Techniques in the preparation of nucleic acids

Modified Thomson and Dietzgen (1995) extraction protocol

Approximately 2mg of sugarcane leaf tissue was macerated in 100µL of template preparation solution (TPS; 100mM Tris-HCl, 1M KCl, 100mM EDTA). The homogenate was boiled for 10mins and quenched on ice. Following a low speed spin, the supernatant was used undiluted as PCR template.

Total Nucleic Acid Extraction of planthoppers (Robertson *et al.*, 1991)

Individual planthoppers were ground in 500uL of extraction buffer (100mM glycine pH 9.5, 100mM NaCl and 10mM EDTA) using a micropestle. The extract was emulsified with an equal volume of phenol by vortexing. The upper aqueous phase was collected after centrifugation at 13000rpm for 15 minutes and precipitated by the addition of 0.1 volume of 3M NaOAc pH 5.2 and 2 volumes of 100% ethanol. The pellet was collected by centrifugation at 13000rpm at 4^{o}C for 30 minutes, washed in 70% ethanol and dried under vacuum. The pellet was resuspended in 50μL of sterile water and stored at –80^{o}C.

Preparation of dsRNA from FDV-infected sugarcane (Dale *et al.,* 1986)

Approximately 10g of FDV-infected sugarcane gall tissue was frozen in liquid nitrogen and ground to a fine powder using a blender. Forty milliliters of extraction reagent (1X STE; 50mM Tris-HCl pH 7.5, 0.1M NaCl, 0.01M EDTA), 20ml of saturated phenol pH 8.0, 20mL of chloroform, 5mL of 10% SDS and 0.5mL 2-mercaptoethanol were added and the mixture stirred for 1 hour at 4^{o}C. The mixture was vacuum filtered to remove particulate leaf material, the filtrate centrifuged at 8000rpm for 20 minutes and the aqueous phase collected. Absolute ethanol was added to the aqueous phase to a final concentration of 17.5%. Whatman CF-11 cellulose powder was added at a concentration of 15% w/w of original plant material and the mixture stirred slowly for 1 hour at room temperature. The cellulose was placed in a small chromatography column and washed free of ssRNA with 50-100mL of 1X STE/17.5% ethanol at room temperature. The dsRNA was eluted from the cellulose by the addition of 100mL of 1X STE and fractions of 5-10mL collected. The dsRNA fractions were precipitated with 0.1 volumes of 3M NaOAc pH 5.2 and 2.5 volumes of absolute ethanol overnight at –20^{o}C and collected by centrifugation at 9000rpm for 60 minutes at 4^{o}C. Dried pellets were resuspended in 2mL of TE buffer pH 8.0, followed by 1mL of cold absolute ethanol and the dsRNA was collected by centrifugation at 13000rpm at 4^{o}C for 60 minutes. The pellets from each fraction were dried, pooled and resuspended in 50μL of TE buffer pH 8.0. The dsRNA was verified as FDV by electrophoresis on a non-denaturing 10% polyacrylamide gel. FDV dsRNA appears as 10 segments ranging from 1.7kb to 4.4 kb. The gel was run at 200V for 4 hours and stained with silver nitrate (Bassam *et al.,* 1991).

Method for the production of *in vitro* RNA transcripts of SCMV

The coat protein and 3' untranslated regions of an Australian isolate of SCMV was cloned into pPCR-Script™ using the PCR-Script™ Amp Cloning Kit (Stratagene). One clone, pSCR-SCMV.36 was found by restriction enzyme analysis and sequencing to contain the desired insert in the reverse orientation. Subsequently, the T7 RNA promoter was used to synthesise positive sense RNA transcripts.

1. Prepare high quality plasmid suitable for use in RNA transcription using the QIAGEN-tip 100 (Midi) Plasmid Purification Kit (Qiagen)

2. Digest 20µg of plasmid DNA with the restriction enzyme *Eag* I (cuts once in the multiple cloning site of pSCR-SCMV.36, downstream of the insert).

3. Treat *Eag* I reaction (from step 2) with 2.5µg/mL Proteinase K for 30 minutes at 37°C. Dilute to 200µL with RNase-free water, extract twice with RNase-free phenol/chloroform and ethanol precipitate. Resuspend pellet in 20µL DEPC-treated water.

4. Synthesise RNA transcripts from pSCR-SCMV.36 using the Stratagene RNA Transcription Kit. One microgram of pSCR-SCMV.36 DNA was transcribed using T7 RNA polymerase as per manufacturer's instructions.

5. Purify synthetic RNA transcripts using the RNeasy Mini Kit (Qiagen). Incorporate a DNase I step to remove plasmid DNA as per manufacturer's instructions. Elute in 50µL of RNase-free water and store at –80°C.

6. Run 10µL of purified RNA transcripts on a denaturing agarose gel to check if transcripts are full-length (ie. 1236bp).

7. Quantitate RNA using spectroscopy and prepare dilution series for use as standard in SCMV TaqMan assay.

Appendix 4

PCR-ELISA technique for the detection of Cxx

PCR-ELISA detection of ratoon stunting disease (RSD) in sugarcane

A manual prepared for the

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Background

The PCR-ELISA test for ratoon stunting disease (RSD) is based on previous work which has identified signature DNA sequences in the intergenic spacer (ITS) region of genes encoding ribosomal RNA in *Clavibacter xyli subsp. xyli (Cxx).* Oligonucleotide primers and hybridisation probes have been designed based on these sequences to allow the specific detection of *Cxx* using a non-radioactive method.

The PCR-ELISA kit developed by Boehringer Mannheim P/L has been adapted to the *Cxx* system. Primers ITSF#5 and ITSR#5 are used to amplify a 304bp region of the *Cxx* ITS in a modified PCR which allows the incorporation of an antigen (digoxygenin) into the DNA during the reaction. Reaction products are then hybridised, in solution, to a biotinylated DNA probe specific to an internal region of the ITS sequence. The hybridisation products are immobilised onto the surface of a MTP well coated with streptavidin (which has a specific affinity for biotin) and non-specific materials are removed in a series of washing steps. The bound hybrid is detected by an anti-digoxygenin peroxidase conjugate and by use of the colormetric substrate ABTS®.

PCR Master Mix Set-Up

1. Before beginning PCR set-up, work out details of the experiment.

- experiment usually includes: -'ve PCR control,+'ve PCR control (pCLAVI or purified *Cxx*), healthy xylem exudate control, test xylem exudate samples.
- add up required master mix (allowing one extra aliquot for pipetting error).
  eg. for 8 test samples you would need a X12 master mix (ie. -'ve control, +'ve control, healthy control, 8 samples and one extra aliquot to discard).
- use the following table to calculate volumes for the master mix. The supplied template can be photocopied for use in PCR set-up.

	(x1)
sterile water	8.7μl
10X PCR buffer (without $MgCl_2$)	2.0μl
$MgCl_2$	1.2μl
DIG-labeling mix	2.0μl
PVP-40 (10%)	2.0μl
Primer ITSF#5 (25pmol/μl)	1.0μl
Primer ITSR#5 (25pmol/μl)	1.0μl
Taq DNA polymerase (5U/μl)	0.1μl
TOTAL	**18.0μl**

2. Swab laminar flow with 70% ethanol and leave motor on until aliquotting reagents.
   NB. the laminar flow creates aerosols which may cause contamination. The fan may be left running while reagents are thawing but must be switched-off prior to pipetting reagents.

3. (Wearing gloves) Write on the required number of 0.2ml SnapStrip™ tubes.
   Do not write on the lids!

4. Make master mix in a 0.6ml tube in order as appears in set-up (ie. enzyme added last).
   Add reagents to bottom of tube to avoid need to centrifuge.
   See notes on preparation of reagents not supplied with the kit at the end of this section.
5. Pipette mix and aliquot 18μl volumes of master mix into 0.2ml SnapStrip™ tubes - close lids.

6. Add 2μl of sterile water to the -'ve PCR control (this tube is not to be opened again).

7. Put away reagents/equipment; swab down laminar flow with 70% ethanol.

8. Transport tubes to RSD lab. NB. Place tubes in purple rack and return transfer container to PCR lab immediately. If left on bench in RSD lab, transfer rack may become contaminated!

List of Equipment required for PCR Set-Up

pipettes - P2, P20, P200
plugged pipette tips for P2, P20, P200
PCR work-up rack
fine-tip marker
0.6ml tubes (autoclaved)
0.2ml thin-walled SnapStrip™ tubes
gloves
PCR-ELISA DIG Detection Kit (Boehringer Mannheim)
transport rack (to be returned immediately to PCR lab)
discard container for used tips

Preparation of PCR Reagents Not Supplied in the Kit

PVP-40 (10%)	Resuspend 0.1g in 1ml of sterile distilled water
Primer ITSF#5	Dilute 1:70 in sterile distilled water
Primer ITSR#5	Dilute 1:50 in sterile distilled water

Template Addition

1. Add 2μl of template using plugged tips

- use a new tip for each sample
- do healthy samples first
- DO NOT open the PCR -'ve control tube!

2. If necessary, spin tubes to collect reagents at bottom. If the reagents are on the side of the tube the PCR will not be uniform.

Thermal Cycling

Perkin Elmer 9600/9700

Initial Denaturation	94°C	5min	(1 cycle)
Denaturation	94°C	10sec	⎫
Annealing	56°C	20sec	⎬ 35 cycles
Extension	72°C	1min	⎭
Hold	4°C		(indefinite)

- Store at 4°C (short-term <2 weeks) or -20°C (longer term >2 weeks) until detection
  NB. Only open PCR product tubes in RSD lab!

PCR-ELISA Detection

Preparation of Working Solutions

When starting a new kit the control PCR product and it's capture probe need to be reconstituted.

Resuspend *control PCR product* (vial 1) in 100µl sterile water - incubate RT/30 min (SOLN I.)
Resuspend *control capture probe* (vial 2) in 100µl sterile water - incubate RT / 30 min (SOLN II.)

Storage:	SOLN I	-20°C
	SOLN II.	4°C (short term); -20°C (long term)

Other solutions are prepared as below and the volumes vary depending on the experiment.

SOLN IV. *Hybridisation soln*
Dilute biotin capture probe to 7.5pmol/ml in ready to use hybe buffer (vial 4).
Add a maximum of 50µl probe to 1ml hybe buffer.
Avoid foaming! Do not freeze!
Dilute 10µl of control capture probe (SOLN II) in 1ml hybe buffer (vial 4).
Can be stored 4°C / 2 weeks but best prepared fresh.

SOLN V. *Washing solution*
Dissolve 1 tablet (vial 5) in 2L distilled water.
Store 4°C / 6 weeks.

SOLN VI. Anti-DIG-POD working solution.
First resuspend one Anti-DIG-POD conjugate (vial 7) in 250µl sterile distilled water.
Mix carefully at RT for 15 minutes (SOLN III). Store 4°C / 2 months.
Do not freeze!

Dilute required amount of Anti-DIG-POD conjugate (SOLN III) 1:100 in conjugate dilution buffer (vial 6) at least 1hr before experiment. Allow to come to RT before use.
Avoid foaming! Do not freeze! Store away from light!
Can be stored 4°C / 2 weeks but best prepared fresh.

SOLN VII. ABTS® - substrate solution
Dissolve one tablet (bottle 9) in 5ml substrate buffer (vial 8).
Allow to come to RT before use.
Store protected from light!
Can be stored up to 3 months at 4°C but best prepared weekly.

PCR-ELISA Detection

Procedure

1. Spin down the PCR products to collect condensation.

2. Label one sterile 1.5ml Eppendorf tube for each sample to be tested. Also label three tubes for (1) a CLAVA (*Cxx)* probe -'ve detection control, (2) a +'ve (human) detection control and (3) a -'ve (human) detection control.

3. Take 5μl of each *Cxx* PCR product to be tested (including -'ve PCR, +'ve PCR and healthy controls) into their respective tubes. Take 10μl human control PCR product (SOLN I) for the +'ve detection control and 10μl sterile distilled water each for the -'ve human probe and -'ve CLAVA probe detection controls.

4. Add 40μl of denaturation solution (vial 3) to each reaction tube. Mix, centrifuge briefly and incubate for 10 minutes at room temperature.

5. Resuspend CLAVA probe to 7.5pmol/ml in hybe buffer (vial 4) = SOLN IV

   NB. You will need 455μl for each *Cxx* sample to be tested and 455μl for the CLAVA -'ve detection control. Remember to make extra hybe soln to allow for pipetting error.

   The supplied diluted CLAVA probe is at 15.5pmol/μl. To make more probe of this concentration dilute the concentrated (~800pmol/μl) probe 1:50 in sterile distilled water.

   An example calculation for hybe solution preparation for 12 samples (including CLAVA -'ve detection control):

   - 455μl (volume of hybe soln per sample) X 12 (no. of samples) = 5460μl
   - Round-up to 6ml to allow for pipetting error and easier calculation
   - Want final concentration of 7.5pmol/ml, therefore, 7.5 X 6 = 45
   - That is, we need to add 45pmol of CLAVA in 6ml of hybe buffer (vial 4)
   - Stock CLAVA is at 15.5pmol/μl, therefore 45/15.5 = 2.9
   - Therefore, add 2.9μl of stock CLAVA (15.5pmol/μl) to 6ml of hybe buffer (vial 4)

6. Add 10μl of control (human) capture probe (SOLN II) to 1ml of hybe buffer (vial 4) to use for +'ve and -'ve detection controls.

7. Add 455µl of CLAVA hybe solution to each of the test samples and 450µl of CLAVA hybe solution to the CLAVA -'ve detection control in 1.5ml tubes. Add 450µl of control (human) hybe solution to +'ve (human PCR product) and -'ve (water) detection controls in 1.5ml tubes. Vortex mix and take 200µl of each into wells of a MTP strip. Incubate 37°C / 3 hours / shaking. **Fill out MTP layout sheet!**

8. Dilute Anti-DIG-POD (SOLN III) 1:100 in conjugate dilution buffer (vial 6) at least 1 hour before start of experiment (SOLN VI). You will need 200µl for each sample (including all controls). Allow extra for pipetting error. Sit at RT for 30 minutes before use.

9. Wash wells 5 times with 250µl of wash solution (SOLN V) flicking contents into sink between washes. After last wash, tap wells dry on lint-free paper.

10. Add 200µl of Anti-DIG-POD working solution (SOLN VI from step 7) to each well. Incubate 37°C / 30 minutes / shaking.

11. Prepare ABTS®: resuspend 1 tablet (bottle 9) in 5ml substrate buffer (vial 8). Prepare enough for 200µl for each sample (including all controls). Allow extra for pipetting error. Allow to come to RT before use.

12. Wash wells 5 times with 250µl of wash solution (SOLN V) flicking contents into sink between washes. After last wash, tap wells dry on lint-free paper.

13. Add 200µl ABTS® (from step 11) to each well and to an empty well (to measure the intrinsic extinction of the substrate). Incubate 37°C / 30 minutes / shaking in dark.

14. Read absorbance on ELISA plate reader at 420nm (reference filter 655nm).

Appendix 5

TaqMan protocols for the detection of Cxx, FDV and SCMV

Note that protocols describe 50 □L reaction volumes in 0.2 mL PCR tubes.

Reaction volumes can be scaled down to 25 □L final volume by halving reagent volumes and adjusting the volume of nuclease-free water.

Sample volumes are generally 1 or 2 □L, but this can be varied for convenience depending on levels of substances with the potential to inhibit reverse transcriptase or thermostable DNA polymerase activity.

General Procedures for TaqMan PCR Set-up

NB. Everything in PCR room is to be handled wearing gloves!

1. Perform calculations for PCR before entering PCR room (See set-up template).

2. Place the following items in laminar flow:
   - Pipettors (P1000, P200, P20, P2)
   - Plugged tips (P1000, P200, P20, P2)
   - Sterile tube for master mix
   - Tube rack
   - Sterile water
   - Primers
   - Probe
   - Baseplate
   - Tray/retainer (set up with tube layout required)
   - Optical lids (inside facing up)

3. UV irradiate for 15-20 minutes (now is the ideal time to set up plate run on computer)

4. Set up PCR master mix, adding reagents in the order as they appear in the template sheet. Always add enzyme last.

5. Dispense 24μL aliquots (for 25μL reactions) and 49μL aliquots (for 50μL reactions) into optical tubes.

5. Add 1μL water to negative controls and replace cap before removing from PCR room. These negative control tubes are <u>not</u> to be opened again in the general laboratory.

6. Put away equipment and reagents and remove tubes to general laboratory for addition of template.

7. Add 1μL of template to PCR tube using plugged tips and P2 pipettor. Replace lids being careful not to handle excessively (to avoid fingerprints on optical plastic lids) and press down firmly using capping tool.

7. Spin plate in benchtop centrifuge at 3000rpm for 15-20 seconds to ensure all reaction mix is at the bottom of the tube.

8. Place tray/retainer in Model 7700 and start run (*see* Model 7700 Operation Procedure).

Preparation of Stock Reagents for TaqMan PCR Assays

Important points to remember

- To prevent degradation, primers and probes should not be thawed more than 6 times.
- Stocks of primers and probes should be stored aliquotted. In case of PCR contamination, only the current aliquot then needs to be discarded.
- Oligonucleotides are more stable at higher concentrations. Therefore, maintain some stocks undiluted.
- Some primer synthesis companies offer concentration adjustment as a free service. Where possible, take advantage of this service to simplify dilutions later.
- Always use sterile (injectable) water for dilutions and construct mixes using plugged pipette tips. Sterile, screw-capped tubes which are certified DNAse-free, RNAse-free and Pyrogen-free are ideal for storing stocks of oligonucleotides. The O-rings in these tubes prevent loss of stock or concentration change through evaporation.
- Store all primers and probes at –20^{o}C.

Primer/probe working solutions for *Cxx*, FDV and SCMV TaqMan assays

Cxx detection using the TaqMan Universal PCR Master Mix (PE Biosystems)

Primers *Cxx*TaqF1 and *Cxx*TaqR1 = 3μM
Probe CLAVTAQ1 = 5 μM

FDV detection using the TaqMan EZ RT-PCR Kit (PE Biosystems)

Primers FDV727F and FDVTq-R = 10μM
Probe FDV-FAM-7 = 5μM

SCMV detection using the TaqMan Gold RT-PCR Kit (PE Biosystems)

Primers SCMVCPf and SCMVCPr = 10μM
Probe SCMVTaqCP = 5μM

TaqMan EZ RT-PCR Kit Set-up Template

REAGENT	Final Conc.	X1 (50μL)	X ___
RNase-free H_2O	-	21.5	
5X TaqMan EZ Buffer	1X	10	
Manganese acetate (25mM)	3mM	6	
dATP (10mM)	300μM	1.5	
dCTP (10mM)	300μM	1.5	
dGTP (10mM)	300μM	1.5	
dUTP (20mM)	600μM	1.5	
FDV-727F (10μM)	200nM	1.0	
FDV-Tq7R (10μM)	200nM	1.0	
FDV-FAM-1 (5μM)	100nM	1.0	
rTth DNA polymerase (2.5 U/μL)	0.1U/μL	2.0	
AmpErase UNG (1U/μL)	0.01U/μL	0.5	
RNasin	0.4U/μL	1.0	
TOTAL		49μL	

DATE: _____

FILE NAME: _____

EXPERIMENT DESCRIPTION: _____

_____

STANDARD TEMPLATE SOURCE: _____

TEST TEMPLATE SOURCE: _____

NOTES:

_____

_____

_____

TaqMan Gold RT-PCR Kit Set-up Template

REAGENT	Final Conc.	X1 (50μL)	X ___
RNase-free H_2O	-	22.5	
10X TaqMan Buffer A	1X	5	
Magnesium chloride (25mM)	5.5mM	11	
dATP (10mM)	300μM	1.5	
dCTP (10mM)	300μM	1.5	
dGTP (10mM)	300μM	1.5	
dUTP (20mM)	600μM	1.5	
SCMVCPf (10μM)	200nM	1.0	
SCMVCPr (10μM)	200nM	1.0	
SCMVTaqCP (5μM)	100nM	1.0	
AmpliTaq Gold (5U/μL)	0.025U/μL	0.25	
Multiscribe RT (50U/μL)	0.25U/μL	0.25	
RNasin	0.4U/μL	1.0	
TOTAL		49μL	

DATE: _____

FILE NAME: _____

EXPERIMENT DESCRIPTION: _____

_____

STANDARD TEMPLATE SOURCE: _____

TEST TEMPLATE SOURCE: _____

NOTES:

_____

_____

_____

PCR Thermal Cycling Protocols

Thermal cycling protocol for *Cxx* using the TaqMan Universal Master Mix

AmpErase UNG activation	50°C for 2 min	
TaqGold activation	95°C for 10 min	
Denaturation	95°C for 15 sec	45 Cycles
Annealing/extension	60°C for 1 min	
Hold	25°C for 2 min	

Thermal cycling protocol for FDV using the TaqMan EZ RT-PCR Kit

AmpErase UNG activation	50°C for 2 min	
dsRNA denaturation	95°C for 5 min	
Reverse transcription	60°C for 30 min	
Denaturation	95°C for 2 min	
Denaturation	94°C for 20 sec	45 Cycles
Annealing/extension	59°C for 1 min	
Hold	25°C for 2 min	

Thermal cycling protocol for SCMV using the TaqMan Gold RT-PCR Kit

Reverse transcription	48°C for 30 min	
TaqGold activation	95°C for 10 min	
Denaturation	95°C for 15 sec	45 Cycles
Annealing/extension	58°C for 1 min	
Hold	25°C for 2 min	

MODEL 7700 OPERATION PROCEDURE

Step One Design of Plate Layout and Run Parameters

1. Open Sequence Detector System software

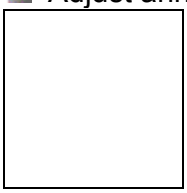
2. Under **File** menu select **New Plate**

3. Check the following are selected
   Plate Type: **Single Reporter**
   Instrument: **7700 Sequence Detector**
   Run: **Real Time**
   Click **OK**

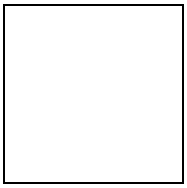
4. Label plate layout using **Sample Type** button. *NB*: If using reporter dyes other than FAM, select the new dye type using **Dye Layer** and nominate new reporters in **Sample Type Setup**
   Add **Sample Name, Replicate and Quantity** if appropriate

5. Set up **Thermal Cycler Conditions**

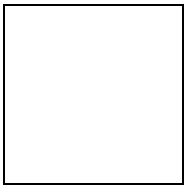
- Ensure UNG activation (50°C/2min) is present for DNA runs
- Ensure TaqGold activation (95°C/10min) is present
- Adjust annealing/extension temperature



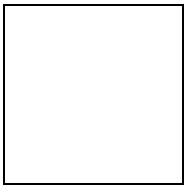
Add a 25°C/2min step at the end of Stage 3 by clicking the mouse once to the right of Stage 3 until a darkened vertical line appears and clicking **Add Hold.** Change temperature of hold from 50°C to 25°C



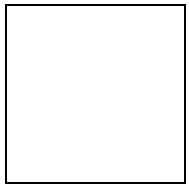
If required, **Add Hold** for reverse transcription stages if necessary



Change number of PCR cycles (Repeat of Stage 3) if required



Press **Show Data Collection** and remove page icons from all steps except anneal/extension phase



Adjust **Sample Volume** if necessary

6. Add a **Comment** if required

7. Save plate using **Save As** under **File**
   Be sure to save plate in current week folder under **SDS Applications**

8. **Quit** from SDS Application Software under the **File** menu and **Restart** the computer under the **Special** menu

Step Two Run Initiation

1. Begin with both the Model 7700 and attached computer switched OFF

2. Turn on Model 7700 at switch on front panel. Wait until green READY light is illuminated before starting computer up

3. Start up computer and open SDS Sequence Detection Software (an empty plate opens up automatically – close this plate)

4. Open saved run file and check details before beginning run

5. Change to analysis view by pressing **Show Analysis/Show Setup** toggle

6. Press **Run** to begin PCR (machine will not start thermal cycling until heated-lid nears 105^{o}C)

7. Wait until run begins before leaving. You should hear a short *beep*, followed by a *click* as the shutter opens and the laser switches on

   NB. If run does not begin or there is an error message, contact an RTPF staff member IMMEDIATELY

8. Check finish time of run and be sure that you have made arrangements for saving of data after run has finished *(Do you have a ZIP disk ready?)*

Step Three Data Download for Analysis

1. The computer automatically imports the run data from the Model 7700 at completion of the run

2. **Save** the plate containing the imported run file and *transfer this file to ZIP disk immediately*. Run files and plates will be deleted from the computer's hard disk drive on the first Monday of every month and no responsibility will be taken for lost files

3. You can also export the data for analysis using Microsoft Excel. Under the **File** menu, select **Export** and **Results** if you wish only to view Cycle Threshold data; select **Clipped Data** if you wish to create amplification plots using Excel (does not include cycle threshold data)

4. The Macintosh computer connected to the Model 7700 is not to be used for detailed data analysis. Similarly, this terminal should not be accessed while a run is in progress. There is another terminal in laboratory 351 that contains the software for interpretation and further analysis of data, however, this computer is not part of the RTPF and priority on this machine is given to the laboratory residents. Regular users should consider installing the SDS analysis software onto their own computers. Please talk to the RTPF about this option.

5. **Printing** RTPF staff are available to provide printing services. Up to 5 pages of black and white printing is included in the run fee; additional pages can be printed at a cost of $0.5 per page. Colour printouts are available for a flat rate of $1.00 per page. If required, RTPF staff can convert amplification plots to Powerpoint Files.