PNNL-13457

Pacific Northwest
National Laboratory
Operated by Battelle for the
U.S. Department of Energy

Nucleic Acid-Based Detection and Identification of Bacterial and Fungal Plant Pathogens

M. T. Kingsley

March 2001

Prepared for the U.S. Department of Energy
under Contract DE-AC06-76RL01830

PACIFIC NORTHWEST NATIONAL LABORATORY
operated by
BATTELLE MEMORIAL INSTITUTE
for the
UNITED STATES DEPARTMENT OF ENERGY
under Contract DE-AC06-76RL01830

Printed in the United States of America

PNNL-13457

Nucleic Acid-Based Detection and Identification of Bacterial and Fungal Plant Pathogens

Final Report

M. T. Kingsley

March 2001

Final Project Report Prepared by M. T. Kingsley for Dr. John Podlesny, Federal Bureau of Investigation, Hazardous Materials Response Unit.

Pacific Northwest National Laboratory
Richland, Washington

EXECUTIVE SUMMARY

This report summarizes the project "Nucleic Acid-Based Detection and Identification of Bacterial and Fungal Plant Pathogens" and TaqMan™ polymerase chain reaction (PCR) assays for three diverse crop pathogens, one bacterial and two fungal. An assay for *Xanthomonas axonopodis pv. citri* A, causative agent of bacterial canker of citrus (a pathogen with significant quarantine restrictions, with a current outbreak in Florida), was the first assay created under this project. A USDA-developed assay for Karnal bunt of wheat, a fungal pathogen of significant quarantine and trade importance, was adapted to the Idaho Technology LightCycler 32. The development of a TaqMan assay for the fungal pathogens *Fusarium graminearum and Gibberella zeae,* significant pathogens of corn, wheat, and other small grains, was initiated, but not completed, within the time frame of the project. The specifics of each of these assays are described in separate sections of this report. This report also describes the facilities at Pacific Northwest National Laboratory that allow us to work with these pathogens and to pass USDA-APHIS permitting requirements, and provides instructions for and a copy of the APHIS permit document form (PPQ-526).

An early deliverable for this project was submission of a Technical Requirements Document that:

(a) identifies species and strains of agricultural pathogens that are forensically significant,
(b) identifies existing PCR assays for the identified agricultural pathogens,
(c) identifies those agricultural pathogens for which PCR assays do not exist or are not adequate
(d) provides recommendations for the development of needed new assays and the validity testing of new and existing assays.

Information contained in the Technical Requirements Document and this Final Report are drawn exclusively from unclassified sources, many of which describe in detail the susceptibility of particular crops to agricultural pathogens.

Presently, more TaqMan assays exist for the bacterial plant pathogens than for the fungal plant pathogens, although that is changing. A number of the fungal plant pathogens will require a significant amount of effort to produce unique PCR assays, which then can be adapted to TaqMan.

Plant pathogenic viruses have not been dealt with in this project, despite the expectation that many viruses are potential threats. Viruses are generally transmitted via insect vectors. In many cases, the appropriate host insect does not exist in the United States. However, for a number of viruses, insects that are "close enough" to serve as vectors do exist in the United States and can serve as disseminators. Although fungal disease can be controlled (oftentimes) through the use of the various fungicides, and some bacterial disease can be controlled through the use of copper-containing sprays, there exist no good chemical-control measures for viruses. From that standpoint, viruses can present an even greater challenge than the bacterial or fungal pathogens.

ACRONYMS

6-FAM	6-carboxyfluorescein
ABI	Applied Biosystems
APHIS	Animal and Plant Health Inspection Service
ARS	Agricultural Research Service
BLAST	basic local alignment search tool
CFU	colony forming unit
Ct	crossing threshold
DIG	digoxygenin
DNA	deoxyribonucleic acid
DON	tricothecene mycotoxin deoxynivalenol
FBI	Federal Bureau of Investigation
FDWSL	Foreign Disease Weed Science Laboratory
FITC	fluorescein isothiocyanate
FRET	fluorescence resonant energy transfer
HEPA	high efficiency particulate air
HIV	human immunodeficiency virus
HMRU	Hazardous Material Response Unit
IT/LC32	LightCycler 32 first sold by Idaho Technology
KB	Karnal bunt
LB	Luria broth
LC32	LightCycler 32
MOPS	3[N-morpholino]propanesulfonic acid

MTA	material transfer agreement
NA	nucleic acid
NCBI	National Center for Biotechnology Information
ntc	no template control (negative control)
PCR	polymerase chain reaction
PNNL	Pacific Northwest National Laboratory
PPQ	Plant Protection and Quarantine
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
R/LC32	LightCycler 32 licensed by Roche
SYBR	(a trade name dye)
TAMRA	carboxytetramethylrhodamine succinimidyl ester
TOPO	topoisomerase (product trade name)
TY-MOPS	tryptone yeast extract-MOPS buffer medium
USDA	U.S. Department of Agriculture
UV	ultraviolet

Table of Contents

Table of Tables

Table of Figures

1.0 Introduction

The threat to American interests from terrorists is not limited to attacks against humans. Terrorists might seek to inflict damage to the U.S. economy by attacking our agricultural sector. Infection of commodity crops by bacterial or fungal crop pathogens could adversely impact U.S. agriculture, either directly from damage to crops or indirectly from damage to our ability to export crops suspected of contamination. Recognizing a terrorist attack against U.S. agriculture and to be able to prosecute the terrorists is among the responsibilities of the members of Hazardous Material Response Unit (HMRU) of the Federal Bureau of Investigation (FBI).

Using polymerase chain reaction (PCR) amplification techniques to analyze the nucleic acids of plant pathogen strains is a powerful method for determining the exact identity of pathogens, as well as their possible region of origin. This type of analysis, however, requires that PCR assays be developed specific to each particular pathogen strain and analysis protocols developed that are specific to the particular instrument used for detection.

The objectives of the work described here were threefold: 1) to assess the potential terrorist threat to U.S. agricultural crops, 2) to determine whether suitable assays exist to monitor that threat, and 3) to determine where assays are needed for priority plant pathogen threats to modify or develop those assays for use by specialists at the HMRU.

The assessment of potential threat to U.S. commodity crops and the availability of assays for those threats were described in detail in the Technical Requirements Document (Kingsley 2000) and will be summarized in this report. This report addresses the development of specific assays identified in the Technical Requirements Document and offers recommendations for future development to ensure that HMRU specialists will be prepared with the PCR assays they need to protect against the threat of economic terrorism.

In addition, a separate project conducted by Dr. Paul Keim and colleagues at Northern Arizona University was included, for funding purposes, under the umbrella of the crop-pathogen PCR project. This project was a research and development project to determine if various chloroplast deoxyribonucleic acid (DNA) sequences from different grass species could be employed as forensic markers for items such as grass stains. A separate report, an assay manual, was prepared by Dr. Keim and submitted to the Bureau. For reporting

purposes, a synopsis of Dr. Keim's research findings, taken from his assay protocol manual, is provided as APPENDIX 7.

2.0 Summary of Research

2.1 Threat Assessment

2.1.1 Threat Review

In the first stage of this work, U.S. commodity crops were reviewed and ranked based on their production value. Significant crop pathogens were identified from open literature sources and by consulting with experts in these crops and pathogens. Among the sources reviewed to assess the threat include the Australian Group Control List of Plant Pathogens for Export Control. Experts consulted included plant pathologists from the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) and the Animal and Plant Health Inspection Service (USDA/APHIS), who are responsible to protect U.S. agriculture with authority for imposition of quarantine. Several university experts were also consulted. The experts who where consulted are listed in Appendix 1. These results were delivered as a Technical Requirements Document.

2.1.2 Jefferson Project

In addition to the threat review conducted as part of this project, Pacific Northwest National Laboratory (PNNL) participated in a panel of experts for the U.S. Department of Agriculture Jefferson Project, whose objective was similarly to identify potential threats to U.S. commodity crops. In October 1999, a group of plant pathologists were assembled at the Battelle Huntsville Alabama office to rank the various pathogens for commodity crops specified by the USDA. The selection criteria provide the greatest weight to 1) toxigenic microbes and 2) those that are easily cultured and 3) are difficult to detect and 4) have the capability to spread rapidly. Obligate parasites, such as the smut and rust fungi, and the tropical downy mildews, which comprise a number of the lists of pathogens of concern, such as the Australia Group List and the CDC Extended List, do not rank very highly when using this set of ranking criteria. However, soybean rust (*Phakopsora pachyrhizi,* which is an obligate parasite) ranked highest for soybean based upon its current quarantine significance to the United States. Soybean rust could induce a major epidemic in U.S. soybeans since limited disease resistance to this pathogen has been bred into the crop. Attached as Appendix

2 is a copy of the Jefferson Project ranking template, and the top four ranked pathogens, for each of eight commodity crops, are listed in Appendix 3.

2.1.3 Threat Assessment Support Information

The contact information for these panels of experts was provided at the end of the Technical Requirements Document and is supplied in this report in Appendix 1.

2.2 Assay Identification

Once the key crops and their significant pathogens were identified, the scientific literature was reviewed to identify which pathogens had PCR assays already developed that were suitable for the specific instruments used in the HMRU.

2.2.1 PCR Instruments

Several fluorescence PCR instruments are currently available that would be suitable to identify phytopathogens by their nucleic acids. These include the Applied Biosystems (ABI) 7700, the LightCycler 32 first sold by Idaho Technology (IT/LC32) and now licensed by Roche (R/LC32), the Cepheid Smart Cycler System, and the Bio-Rad I-Cycler. Each of these instruments has particular strengths. For example, the advantage the LC32 has over the ABI 7700 is speed and true "real time" detection, which allows for observing the assay progressing (or not) during a run. While the ABI 7700 claims to provide real-time assay information, it is only provided after the assay is completed and the control program processes the data, and not while the assay is running. The LC32, when it was first introduced, was significantly cheaper than the ABI 7700, and there were no other competing fluorescent PCR machines commercially available. That has changed as Cepheid has introduced their Smart Cycler System fluorescent PCR machine to the market, and Bio-Rad introduced the "i-Cycler." Also, Idaho Technology produced "ruggedized" LC instruments that were amenable to remote use in the field. This was impossible with the very large laboratory instruments such as the ABI 7700.

In addition to the operational differences described above, assays optimized for one instrument type might not work directly in another instrument without some modification, due to differences in physical parameters of sample cell, fluorometer optics, and the specific fluorescent dyes employed.

2.2.2 Types of Assays

One type of fluorescent PCR assay that is commonly recognized is the TaqMan™ assay, originally developed for the ABI 7700 sequence detection instrument. Although developed for the ABI 7700, TaqMan assays can be made to work in the LightCycler if the chemistry of the assays is modified appropriately. The TaqMan assay is based upon the 5' exonuclease activity of certain Taq polymerase enzymes. When the TaqMan probe is intact, fluorescence resonant energy transfer (FRET) prevents the detection of the fluorophore 6-FAM (6-carboxyfluorescein). The 6-FAM fluorescence is blocked by FRET transfer to a second fluorophore TAMRA (carboxytetramethylrhodamine succinimidyl ester), attached at the 3' end of the probe. During PCR amplification, if the appropriate target is amplified, the TaqMan probe (which is complementary to one strand of the target amplicon) anneals to the template. The 5' exonuclease activity of Taq polymerase cleaves the probe. This alleviates FRET blockage of the 6-FAM fluorescence, and the fluorometer is capable of detecting an increase in 6-FAM fluorescence, which is detected in the FITC (fluorescein isothiocyanate) channel of the LC32.

2.2.3 TaqMan Assay

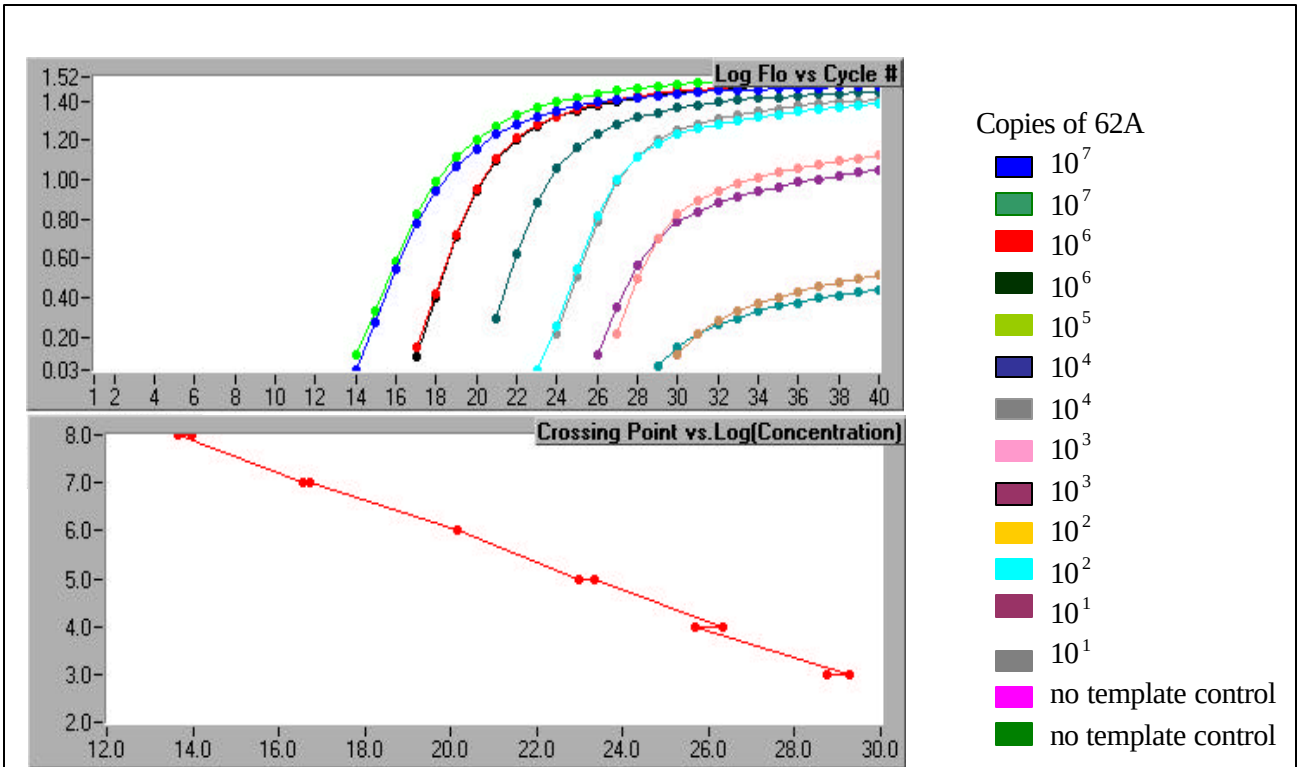
TaqMan assays are powerful. They provide significant information about the quantity of a target in a particular nucleic acid sample. In clinical applications, in addition to TaqMan's capability to specifically identify whether a pathogen (or any desired nucleic acid (NA) target is present, TaqMan assays are routinely employed to determine the titer of a pathogen in blood, for example. This is because the greater the target concentration in the sample, the more the TaqMan probe anneals initially and is cleaved during the early cycles of PCR amplification. Therefore, the fluorescence of high titer samples increases rapidly over the threshold and is detected by the fluorometer earlier in the assay than in samples with lower copies of the target. The higher the initial target concentration, the more rapidly fluorescence is detected. Conversely, lower target concentrations require various cycles of PCR to amplify the target sufficiently to the point that enough TaqMan probe is cleaved, and fluorescence is detected by the fluorometer (see Figure 1 for example, which illustrates the effect of target concentration on the rate at which fluorescence is detected). The higher the target concentration, the fewer cycles of PCR are required for detection. The crossing

threshold (Ct), in conjunction with a standard curve (dilution series of a standard sample of known quantity) can be employed to quantify targets in a sample, for example, the human immunodeficiency virus (HIV) load in a patient's blood sample or the quantity of a phytopathogen in a plant-tissue sample.

In determining whether PCR assays were already available for use by HMRU to monitor various phytopathogens, the pathogens were characterized as to whether no PCR assay development had been found, assays had been developed but were gel based, or an appropriate (TaqMan) assay had been developed.

2.3 Assay Development

To make the PCR assays immediately transferable to HMRU laboratory scientists, the assays were developed specifically for the LC32 from Idaho Technology Incorporated. The LC32 was purchased under this project for developing and testing these assays. As noted above, however, with appropriate modification of the chemistry, TaqMan assays developed for the ABI 7700 will work in the LC32. Here will be described 1) the development of a TaqMan and dual FRET probe assay for *Xanthomonas axonopodis* pv. citri, the bacterial pathogen that causes citrus canker, currently a significant phytopathogen of concern in Florida, 2) the adaptation to the LightCycler of a TaqMan assay for Karnal bunt of wheat (*Tilletia indica*), developed by Dr. Reid Frederick of the USDA-ARS FDWSL (Foreign Disease Weed Science Laboratory) for the ABI 7700, and (3) the development of a TaqMan assay for *Fusarium graminearum/Gibberella zeae,* fungal pathogens causing diseases of wheat, other small grains, and of corn.



Sensitivity test of X. citri taqman assay. *This figure illustrates the effect of DNA target concentration ranging from 10^7 copies TL/assay to 10^1 copies TL/assay (see key for sample color coding). The higher the concentration, the more rapidly sample fluorescence increases. The upper panel plots the log of fluorescence against cycle number (1-40 cycles). The lower panel plots the crossing point against the log of the DNA concentration. For a standard curve, a straight line, like the one seen, is desired. By comparing the Ct of an "unknown" sample with the standard curve, one can estimate the concentration of the particular target in the sample. The LightCycler software will do this for you automatically. The DNA source in this example is X. citri. Strain 62 genomic DNA.*

Figure 1. *Xanthomonas citri* TaqMan Assay Standard Curve

2.3.1 USDA-APHIS Permitting and Culture Handling Requirements

The USDA APHIS regulates all work with plant pathogens and requires special permits to transport, receive, or to do any work with phytopathogens. The document known as "PPQ 526" (Plant Protection and Quarantine) permits application is provided as Appendix 4 along with the appropriate instructions. The full two-page permit may be downloaded from the APHIS web site (http://www.aphis.usda.gov/ppq/ss/permits/pests/), and a new, online permit application process for importing pathogens form outside the continental United States has been instituted (https://web01.aphis.usda.gov/IAS.nsf/Mainform?OpenForm). In a PPQ-526 permit application, the user must describe the pathogen, its form, methods for control/prevention of escape, the intended experimental use, and final method(s) of disposition (see Appendix 4 for specifics). For pathogens of significant quarantine concern, for example *Xanthomonas citri*, APHIS requires descriptions of the laboratory facilities where the experiments are to be conducted. Specifically, APHIS wants to know what types of controls are in place to prevent the escape of the pathogen into the environment. For example, at PNNL, we have high efficiency particulate air (HEPA) filtered environmental growth chambers contained within a secured, restricted access basement laboratory. The air exiting the growth chamber passes through the HEPA that removes small micron and sub micron-sized particles. Additionally, the direction of airflow is from the outside of the laboratory towards the inside (therefore keeping potential airborne contamination within the confines of the laboratory airspace), and all air exiting the laboratory passes through a HEPA filter bank before being exhausted to the atmosphere. This ensures that no pathogens will inadvertently "escape up the stack." Also, all potentially contaminated materials must be double or triple bagged and autoclaved for decontamination before disposal. These more restrictive rules apply to plant infection tests with *X. citri*. In fact, while the Washington State Department of Agriculture would have allowed us to conduct *X. citri* host infection tests in our open (non-containment) greenhouses, APHIS required that we conduct all *X. citri* host infection tests in the secured (restricted access, HEPA filtered) basement laboratory growth chamber. The permitting requirements are less restrictive if simply growing the organisms for isolation of their nucleic acids, although this can vary with the "exoticness" of the particular pathogen. The permitting requirements (facilities required, etc.) will vary significantly with the pathogen and the region of the country in which the work is to be

performed. All PPQ-526 applications require approval by the state agricultural department and ultimate approval by USDA-APHIS in Riverdale, Maryland. For this HMRU-sponsored project, we have one permit for the *Xanthomans* studies and a separate permit for the *Fusarium graminearum/Gibberella zeae* studies. The Fg/Gz permit is currently restricted to culturing the pathogens for DNA isolation only. We would need an amendment to perform host infection tests. For *Tilletia indica* (Karnal bunt), we only worked with DNA samples provided to us; the pathogens (and their permits) were maintained at the USDA Foreign Disease Weed Science Laboratory at Ft. Detrick, MD, so a PNNL-specific permit was not required.

2.3.2 PNNL Plant Growth Facilities

There are four greenhouses with a combined total of 4800 ft^2 of year-round growing space. The greenhouses are equipped with heating and cooling devices as well as supplemental lighting that includes a mixture of fluorescent, metal halide, and mercury vapor fixtures. There are also four growth chambers, three 32 ft^2, and one 12 ft^2, all with temperature and humidity control. Lighting includes a mixture of fluorescent and incandescent sources to provide an illumination intensity of >400 µmol m^{-2} s^{-1} (400 to 600 nm) at bench level.

2.3.3 Culture Preservation and Handling

Xanthomonas cultures (turbid, overnight, broth cultures) were stored as frozen glycerol stocks (15% glycerol final concentration) at -80°C (Queue Cryostar freezer) in sterile 2 mL screw-cap cryovials (Sigma or Nunc) (Gabriel et al. 1989). Fungal cultures were stored at 4°C over dry, sterile silica gel as described in the *Fusarium* section of the Methods for Research on Soilborne Phytopathogenic Fungi (Windels 1992). *Fusarium* cultures may also be stored by lyophilization (Windels 1992). All culture handling was performed in Class II biosafety cabinets (The Baker Company, Sanford, Maine).

3.0 Modification and Development of Assays

3.1 Xanthamonas axonopodis pv. citri

3.1.1 Background

Asiatic bacterial canker of citrus, induced by *Xanthomonas axonopodis* pv. citri A (*X. citri* A, XacA), has re-emerged as a threat with significant impact to the Florida citrus industry. Strong regulatory and quarantine statutes are invoked when the pathogen has been identified in U.S. citrus. These statutes mandate the clearing and elimination by burning of infected and nearby trees (Gottwald et al. 2001). Past experience reinforces the need for accurate and specific identification of the causative organism: in 1984, a "canker" outbreak was incorrectly identified in nursery stock, primarily Single citrumelo (*Citrus paradisi* X *C. trifoliata*). This disease was later determined to not be canker and therefore fell outside of the regulatory statutes. This group of strains, currently designated as *X. axonopodis* pv. citrumelo, which cause the disease citrus bacterial spot, is considered of little economic importance (Verniere et al. 1998). A number of lawsuits arose in Florida as a result of the misidentification and resultant unnecessary loss of crops.

As discussed recently by Verniere et al. (Verniere et al. 1998), there are a number of xanthomonads associated with disease of citrus. Asiatic canker, or A strain, is the most widely distributed geographically and is the most important economically since it has the broadest range of citrus hosts (grapefruit, oranges, tangelos, pomelos, limes, lemons, tangerines, and other citrus fruits). Canker is thought to have originated in Southeast Asia, and its geographic spread is increasing, despite strict quarantine regulations imposed by many countries (Whiteside et al. 1988). Grapefruit, key lime, and trifoliate orange are generally considered most susceptible to Asiatic canker (Whiteside et al. 1988). Another xanthomonad "Cancrosis B" (*X. axonopodis* pv. citri pathotype B, Xac-B) is found in a few countries in South America with a host range primarily limited to lemons (*Citrus limon*). Mexican lime disease is caused by an even more delimited xanthomonad. It is found in Brazil and only affects Mexican lime (*C. aurantiifolia* [Christm.] Swingle); the causative agent is currently designated Xac pathotype C (Xac-C). Another series of strains recently isolated from Southwest Asia (Verniere et al. 1998), while genotypically similar to XacA,

has a proscribed host range more analogous to the C pathotype. These strains are similar to *X. citri* A strains in their hydrolysis of casein and gelatin, growth on 3% NaCl, by genomic restriction fragment length polymorphism (RFLP) patterns, DNA probe (dot blot) reactions, and PCR for an XacA plasmid-borne target (Verniere et al. 1998). In addition to their host-range limitations, these strains varied from XacA strains by Biolog® analysis, differences in bacteriophage susceptibility, and monoclonal antibody reaction (Verniere et al. 1998). The A* strains represent yet another variant of xanthomonads that attack citrus; the genetic basis for the differences between A and A* strains are currently unknown. However, as previously mentioned, these strains appear to be homologous to typical XacA by various DNA assays.

While the current taxonomic assignment of *Xac-A* to the species *X. axonopodis* has recently been called into question (Schaad et al. 2000), and the designation to species status of *X. citri* by Gabriel et al (Gabriel et al. 1989) was rejected (Young et al. 1991), the need to rapidly and accurately identify the pathogen, irrespective of its ultimate taxonomic designation, still exists. Currently, $175M is targeted towards canker eradication in Florida; of this, approximately $8M is to be used to fund new research on citrus canker detection, control strategies, and the development of host resistance (Gottwald et al. 2001).

Fluorescent PCR analyses with internal FRET identification probes, either hydrolysis probes (i.e. TaqMan™) or dual FRET probes (Lay and Wittwer 1997; Wittwer et al. 1997) diagnostic for the target of interest, provide for real-time detection and identification. Such approaches conserve significant amounts of operator time since separate gel-based or other off-line detection schemes for PCR products are not required. Since fluorescence only results if the complimentary target sequence is amplified, the needs for other forms of product validation are eliminated. Amplification, detection, and identification occur simultaneously in one step. Fluorescent PCR analyses for the Asiatic bacterial pathogen *Xanthomonas axonopodis* pv. citri A should significantly reduce the time required to identify this pathogen of significant quarantine importance.

We have developed two rapid fluorogenic PCR assays (TaqMan, dual FRET probe), which detect and identify *Xanthomonas axonopodis* pv. citri A (XacA, *X. citri*) strains, the causal agent of Asiatic citrus canker, in extracts from citrus leaf tissues. Each assay is based upon the same forward and reverse primers that amplify a 126 base pair (bp) chromosomal target. The 126 bp target amplicon, derived from a unique random amplified polymorphic

DNA (RAPD) fragment, was diagnostic for *X. citri* A and A* strains from diverse geographic regions, by dot-blot hybridizations, Southern blot hybridizations, and PCR. There is no significant homology of this target (or of the full-length RAPD fragment), as determined by the basic local alignment search tool (BLAST) analysis, to anything currently present in GenBank.

The TaqMan assay requires a polymerase with 5' exonuclease activity, while the dual FRET probe assay requires a nuclease-free polymerase (KlenTaq) and the phoshorylation of the 3' end of the 3' probe. The assay(s) detected 38 of 38 XacA strains tested and did not amplify any of a broad array of control DNA samples tested. The 40-cycle, single-tube assay can be monitored in real time and provides an answer within 20 min when using the LightCycler. The total time required for an analysis, from tissue punch through to positive or negative PCR results is ≤4 h. A detection level of approximately 10^2 targets/reaction was achieved with DNA from pure XacA cultures, and approximately 10^3 targets/reaction sensitivity was achieved when XacA-infected citrus leaf extracts were analyzed. The assay does not detect other *Xanthomonas* pathogens affecting citrus (*X. axonopodis* pv. aurantifolii strains B or C [cankrosis B, Mexican lime bacteriosis, respectively], *X. axonopodis* pv. citrumelo [citrus bacterial spot]). Considering the problems the Florida citrus industry is currently facing, the specificity and sensitivity of the assay, combined with high speed, offer the potential to screen citrus grove materials for the presence of the pathogen and so may prove useful from a quarantine standpoint.

3.1.1.1 Bacterial Strains and Plasmids, Culture Conditions, and Sources of DNA

Escherichia coli, *Xanthomonas*, plasmids, other heterologous DNA samples, PCR primers and fluorescent probes used in this study, their sources, and references are listed in Table 1. *Xanthomonas* was cultured in tryptone yeast extract-MOPS buffered (TY-MOPS) (Gabriel et al. 1988) broth and checked for purity and uniformity on TY-MOPS agar plates incubated at 30°C. Handling and long-term storage of the cultures was as described previously (Gabriel et al. 1989). Cultures of *E. coli* were grown at 37°C in Luria broth (LB) and on LB-agar plates and were maintained and stored according to standard protocols (Sambrook et al. 1989). All PCR primers were synthesized commercially (Genosys Biotechnologies, Inc., The Woodlands, Texas). TaqMan™ hydrolysis probes were synthesized by ABI (PE Biosystems Inc., Foster City, California) and incorporated 5' 6-FAM

(6-carboxyfluorescein) and 3' TAMRA (carboxytetramethylrhodamine succinimidyl ester) fluorescent dye labels. Dual FRET probes were synthesized by Operon (Operon Technologies, Inc., Alameda, California) by incorporating a 3' fluoroscein at the terminus of the 5' probe, and a Cy5 (cyanine) molecule on the 5' end of the 3' probe; the 3' end of the 3' probe was phosphorylated to prevent extension (Lay and Wittwer 1997; Wittwer et al. 1997).

Table 1. Bacterial Strains, Plasmids and Sources of DNA Used in this Study

Bacterial strain, plasmid, other source of DNA	Relevant Characteristics	Source or Reference
Xanthomonas axonopodis		
pv. axonopodis ATCC 19312[T]	Type Strain	ATCC
pv. citri A strains		
3210	Florida	(Gabriel et al. 1989)
3213	Florida	(Gabriel et al. 1989)
Xc 62	Japan	(Verniere et al. 1998)[1]
Xc 100	Pakistan	(Verniere et al. 1998)
Xc 158	Pakistan	(Verniere et al. 1998)
Xc 164	India	(Verniere et al. 1998)
Xc 165	India	(Verniere et al. 1998)
Xc 167	India	(Verniere et al. 1998)
Xc 168	India	(Verniere et al. 1998)
Xc 170	India	(Verniere et al. 1998)
Xc 251	Yemen	J. Hartung
JF90-5	Oman	(Verniere et al. 1998)
JF90-8	Oman	(Verniere et al. 1998)
JF90-12	Oman	(Verniere et al. 1998)
A* strains		
Xc 166	India	(Verniere et al. 1998)
Xc 169	India	(Verniere et al. 1998)
Xc 269	Saudi Arabia	(Verniere et al. 1998)
Xc 271	Saudi Arabia	(Verniere et al. 1998)
Xc 273	Saudi Arabia	(Verniere et al. 1998)
Xc 274	Saudi Arabia	(Verniere et al. 1998)
Xc 275	Saudi Arabia	(Verniere et al. 1998)
Xc 276	Saudi Arabia	(Verniere et al. 1998)
Xc 277	Saudi Arabia	(Verniere et al. 1998)
Xc 278	Saudi Arabia	(Verniere et al. 1998)
Xc 279	Saudi Arabia	(Verniere et al. 1998)
Xc 280	Saudi Arabia	(Verniere et al. 1998)
Xc 282	Saudi Arabia	(Verniere et al. 1998)
Xc 283	Saudi Arabia	(Verniere et al. 1998)
Xc 289	Saudi Arabia	(Verniere et al. 1998)
Xc 290	Saudi Arabia	(Verniere et al. 1998)
Xc 291	Saudi Arabia	(Verniere et al. 1998)
Xc 292	Saudi Arabia	(Verniere et al. 1998)
Xc 293	Saudi Arabia	(Verniere et al. 1998)
Xc 322	Saudi Arabia	(Verniere et al. 1998)
Xc 323	Saudi Arabia	(Verniere et al. 1998)
Xc 328	Saudi Arabia	(Verniere et al. 1998)

Bacterial strain, plasmid, other source of DNA	Relevant Characteristics	Source or Reference
Xc 329	Saudi Arabia	(Verniere et al. 1998)
JF90-2	Oman	(Verniere et al. 1998)
JF90-3	Oman, Xc206	(Verniere et al. 1998)
JM47-2	Iran	(Verniere et al. 1998)
pv. aurantifolii		
B strains		
Xc 64	Argentina	(Verniere et al. 1998)
Xc 69	Argentina	(Verniere et al. 1998)
Xc 84	Uruguay	(Verniere et al. 1998)
C strain		
Xc 70	Brazil	(Verniere et al. 1998)
D strain		
XC90	Mexico	(Verniere et al. 1998)♦
X. axonopodis		
pv. alfalfae		
KX-1	KS-1	(Lazo et al. 1987)
82.1		(Lazo et al. 1987)
L334		(Lazo et al. 1987)
L676		(Lazo et al. 1987)
pv. citrumelo		
3048		(Lazo et al. 1987)
pv. cyamopsodis		
13D5		(Gabriel et al. 1989)
pv. dieffenbachiae		
729		R.E. Stall[2]
2032		R.E. Stall
pv. malvacearum		
N		(Lazo et al. 1987)
Xanthomonas campestris		
pv. campestris		
ATCC 33913[T]	Type strain, NCPPB 528	ATCC, (Gabriel et al. 1989)
X3		(Lazo et al. 1987)
X. oryzae pv. oryzae		
ATCC 35933[T]	Type strain	ATCC*
ATCC 43836		ATCC*
ATCC 43837		ATCC*
X.o. pv oryzicola		
ATCC 49072		ATCC*
X. translucens		
G25		(Lazo et al. 1987)
Other heterologous DNA sources		
Fusarium oxysporum		
f.sp. lycopersici		
ATCC 42323		ATCC
Streptomyces roseoflavus		
ATCC 13167		ATCC
Bacillus thuringiensis		
ATCC 10792[T]	Type Strain	ATCC
Cow		Jim Robertson[3]
Pig		"
Rat		"
Phage Lambda		"
Phage T7		"
Phage T4		"
Potato		
Tobacco		

Bacterial strain, plasmid, other source of DNA	Relevant Characteristics	Source or Reference
Tomato		
Citrus aurantiifolia 'Dwarf Bearss Seedless' Flying Dragon	Bearss seedless lime	Clifton's nursery
Citrus limon 'Meyer'	Meyer lemon	"
Citrus reticulata 'Dancy'	Dancy tangerine	"
Citrus sinensis 'Midnight'	Midnight Valencia orange	"
Citrus sinensis 'Washington Navel'	Washington navel orange	"

[1]Cultures referred to in (Verniere et al. 1998) were obtained from Dr. John Hartung, USDA, Beltsville, Maryland.

*ATCC no longer sells these cultures.

[2]R.E. Stall, U. Florida

♦"D" strain, no longer considered a separate strain, grouped into C strains.

[3]DNA samples courtesy of Dr. James Robertson.

3.1.1.2 Nucleic Acid Methods

High molecular weight chromosomal DNA was isolated from approximately 5-mL of overnight cultures (25 mL cultures) of *Xanthomonas* using Qiagen Genomic-Tip 100/G kits (Qiagen Inc., Valencia, California) according to the manufacturer's protocol. Plasmids from *E. coli* or *Xanthomonas* and chromosomal DNA for PCR reference obtained from other bacteria or fungi were isolated by standard methods (Sambrook et al. 1989). Citrus DNA was isolated from approximately 2-week-old citrus leaves using a Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, California) according to the manufacturer's protocol. Non-radioactive nucleic acid probes for Southern hybridization were labeled by incorporating digoxygenin (DIG)-labeled nucleotides by random priming or PCR amplification protocols (Roche Molecular Biochemicals, Indianapolis, Indiana). Standard methods were used for Southern transfer of DNA from agarose gels to nylon membranes (MSI Magnagraph, Micron Separations, Inc., Westboro, Massachusetts) (Sambrook et al. 1989). DIG-labeled probes were washed from blots following high-stringency washes (Sambrook et al. 1989), and the probes were detected with the biotin luminescent detection kit (Roche Molecular Biochemicals, Indianapolis, Indiana). The results were recorded by exposing the blots to X-ray film. All primers for PCR amplifications and cycle sequencing were designed with the aid of MacVector 6.5 (Oxford Molecular Ltd., Oxford, UK). Fluorescent PCR probes (TaqMan and dual FRET) were designed and with the aid of PE Primer Express (PE Biosystems, Foster City, California). RAPD PCR amplification of *Xanthomonas* DNAs was performed using a PE model 480 thermocycler. All other, non-fluorescent DNA amplifications were conducted on Techne Genius thermocyclers equipped with a heated-lid and 96 well, 0.2-mL tube size heat block (ISC BioExpress, Salt Lake City, Utah). Real-time

fluorescent PCR reactions were performed with an Idaho Technology LightCycler 32 (LC32) system (Idaho Technology, Salt Lake City, Utah) set up for dual channel (fluorescein and Cy5) fluorescence analysis. Qiagen Taq DNA polymerase (Qiagen Inc., Valencia, California) was used for standard and TaqMan PCR reactions. Qiagen Taq DNA polymerase has the necessary 5'–3' exonuclease activity to cleave the TaqMan probe. KlenTaq Taq DNA polymerase was purchased from Ab Peptides (St. Louis, MO). KlenTaq is completely exonuclease minus and was used for the dual FRET probe fluorescent PCR reactions in the LC32.

3.1.1.3 Selection of *X. citri* PCR Target Sequence

RAPD primers OPM-1, OPM-2, and OPM-12 (Operon Technologies, Inc. Alameda, California) were found useful for differentiating *X. citri* strains 3210 and 3213 from other xanthomonads (data not shown, H. Parker and M.T. Kingsley, unpublished results). *Xanthomonas* chromosomal DNA was subjected to RAPD analysis using oligonucleotide primers OPM-1, OPM-2, and OPM-12 (Operon Technologies, Inc., Alameda, California) following the protocol outlined with the RAPD primer kits. Following PCR amplification, the reaction products were separated by agarose gel electrophoresis (1.4% gel strength) and visualized by staining with ethidium bromide and ultraviolet (UV) illumination. DNA bands of interest were excised from the gels, and the DNA was recovered from the gel slice with a QIAEX gel extraction kit (Qiagen Inc., Valencia, California). The purified RAPD fragments were labeled with DIG and used as hybridization probes of DNA dot-blots. Serial dilutions of *Xanthomonas* DNA were spotted onto Magnagraph nylon membranes using a dot-blot apparatus (Minifold I, Schleicher & Schuell, Keene, New Hampshire). Probe hybridization and non-radioactive detection were as described above. To clone the RAPD fragments, the individual, purified *X. citri* OPM-1, OPM-2, and OPM-12 RAPD bands were re-amplified by PCR using the corresponding RAPD primers. The fragment ends were polished using *Pfu* DNA polymerase (PCR Polishing Kit, Stratagene, La Jolla, California), and the individual fragments were cloned into pCR-Blunt II-TOPO[(1)] using the Zero Blunt TOPO PCR cloning kit (Invitrogen Corp., Carlsbad, California). The cloned *X. citri* RAPD fragments were sequenced at the DNA Sequencing Facility, Iowa State University (Ames, Iowa). The *X.*

([1]) TOPO=topoisomerase, part of a product trade name.

axonopodis pv. citri TaqMan assay probe (Figure 2) and the dual FRET probe (Figure 3) assays were developed using rules and guidelines recommended by Applied Biosystems or Idaho Technologies, respectively. For TaqMan assays, the forward/reverse primers and probe comply to the suggested parameters for TaqMan probe/primer design: the last five nucleotides of the primer must contain no more than two G/Cs, the probe must contain more C than G, and the 5' base of the probe must not be a G. The sequence and corresponding primer and probe information were deposited as GenBank AF312370 (see Table 2).

TaqMan Assay

TaqMan Probe
6-**FAM**-ACCACAGAAGAGTTCAAGCCGGCATG-**TAMRA**
Forward Primer
5′ TCCACTGCATCCCACATCTG
Reverse primer
5′ CAGGTGTACTGCGCTCTTCTTG

Reaction Conditions
0.5 mM of *X. citri* forward and reverse primers
0.05 mM TaqMan probe
0.2 mM dNTPs
5 mM $MgCl_2$
10 mM Tris-HCl (pH 8.3)
50 mM KCl
0.25 mg/ml BSA
0.5 – 1 unit of Taq DNA polymerase and template DNA
(5'-3' exo+ Taq)*

LightCycler Conditions
Template detection occurs within 40 cycles of:
denature at 94°C for 0 sec, ramp rate of 20°/sec
anneal at 54°C for 0 sec, ramp rate of 20°/sec
extension at 65°C for 30 sec, ramp rate 2°/sec

(Acquisition of the fluorescent signal occurs at the end of each extension cycle excite/read **FAM** fluorescence FRET-blocked by TAMRA until cleaved by Taq exonuclease activity)
*(Qiagen Taq DNA polymerase)

Figure 2 *X. citri* TaqMan Assay

Dual FRET Probe Assay

5' Probe

5' GAGCGTGACATGACCAGGTATAAGGA 3'-**FITC**

3' Probe

5' **Cy5**-CGCACCACAGAAGAGTTCAAGCC 3'-PO_4

Forward Primer

5′ TCCACTGCATCCCACATCTG

Reverse primer

5′ CAGGTGTACTGCGCTCTTCTTG

Reaction conditions

0.50 mM of each primer
0.05 mM of each probe
0.2 mM dNTP
5 mM $MgCl_2$
50 mM Tris-HCl (pH 9.1)
16 mM $(NH_4)_2SO_4$
0.25 mg/ml BSA
8 ng of genomic DNA
1 unit of KlenTaq DNA polymerase .

LightCycler Conditions

Template detection occurs within 40 cycles of:
denature at 94°C for 0 sec, ramp rate 20°/sec
anneal at 54°C for 0 sec, ramp rate 20°/sec
extend at 63°C for 30 sec, ramp rate of 2°/sec

(Acquisition of **red** and **green** fluorescence occurs at the end of each extension cycle **excite FITC, aquire Cy5 fluoresence**)

Figure 3 *X. citri* dual FRET Probe Assay

Table 2. GenBank Submission of the Genetic Information for the X. citri PCR Assay

From gb-admin@ncbi.nlm.nih.gov

==========> bankit361501, Accession No. **AF312370**

Submission 1 of a set of 1 submission(s).

Comment: source of genomic DNA: XacA strain 3210

```
LOCUS       nkit361501   1298 bp    DNA             BCT       10-OCT-2000
DEFINITION  X. axonopodis pv. citri A, strain 3210, OPM-12 SCAR fragment.
ACCESSION   ;
KEYWORDS    .
SOURCE      Xanthomonas axonopodis pv. citri.
  ORGANISM  Xanthomonas axonopodis pv. citri
            Bacteria; Proteobacteria; gamma subdivision; Xanthomonas group;
            Xanthomonas.
REFERENCE   1  (bases 1 to 1298)
  AUTHORS   Kingsley,M.T., Fritz,L.K. and Parker,H.G.
  TITLE
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1298)
  AUTHORS   Kingsley,M.T., Fritz,L.K. and Parker,H.G.
  TITLE     Rapid, real-time LightCycler-based fluorescent polymerase chain
            reaction assays for the detection and identification of Xanthomonas
            axonopodis pv. citri A, causal agent of Asiatic citrus canker
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 1298)
  AUTHORS   Kingsley,M.T., Fritz,L.K. and Parker,H.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-OCT-2000) ETD, Environmental Characterization and
            Risk Assessment Group, Pacific Northwest National Laboratory,
            POB-999; MSIN K2-21; PSL606, Richland, WA 99352, USA
COMMENT     Bankit Comment: source of genomic DNA: XacA strain 3210.
FEATURES             Location/Qualifiers
     source          1..1298
                     /organism="Xanthomonas axonopodis pv. citri"
                     /strain="3210"
                     /db_xref="taxon:92829"
                     /note="cloned RAPD fragment, OPM-12 primer;"
     primer_bind     436..454
                     /note="Forward PCR primer,   TCCACTGCATCCCACATCT"
     misc_feature    436..561
                     /note="PCR amplicon"
     primer_bind     473..497
                     /note="Dual FRET 5' probe, 3' -FITC fluorescent label;
                     GAGCGTGACATGACCAGGTATAAGG"
     primer_bind     501..523
                     /note="Dual FRET 3' probe,  5'-Cy5 fluorescent label, 3'
                     PO4 blocked;  CGCACCACAGAAGAGTTCAAGCC"
     primer_bind     504..529
                     /note="TaqMan probe sequence, complementary to (-) strand;
                     5' 6FAM fluorescent label, 3' TAMRA fluorescent label;
                     ACCACAGAAGAGTTCAAGCCGGCATG"
     primer_bind     complement(541..561)
                     /note="Reverse PCR primer,   CAGGTGTACTGCGCTCTTCTT"
BASE COUNT      272 a    394 c    400 g    232 t
```

```
ORIGIN
        1 cagatttagg atatgtccca cggctgctga ggcagccagc tgacaaggcg gaagctctc
       61 ccgccgcatt ccgacccttg cacggggtcg taaataacac ggtgcggctg gaccagctaa
      121 ggcgcgatga ggcgtcgttg cgggaagtcc gggacgggaa tggctctgcc ttcacccacc
      181 cacatgacga cccctcaacg acagcggagc ggcactggcc cgaaaccggc acggcctgac
      241 aatcctgcac accgcaggag agtcgggcca ctcaagcgcc tcggtcacac gggggtattt
      301 gagtggcttg attcaatgga gaaccctatg gaacacgatg cgatccccta cgttgcaccc
      361 gacgaccgcc tgatggacac cgtgaggtgg gaactggcgt gctaccggac gagcatcggc
      421 cgtaccgaag ccctgtccac tgcatcccac atctggcaca aatcccacga aggagcgtga
      481 catgaccagg tataaggaag cgcaccacag aagagttcaa gccggcatgg ggaggccggc
      541 aagaagagcg cagtacacct gcctgcgcgt cgttggccgt tgggtgctgc ggcaccacgg
      601 ccttgcggtt ggcatctatc cgactctggg cgcacttgtt gctgcccggc tcgaacacga
      661 aggacgcact catggctgag cacgacggga tcactgaggg gctggagtgc gacaacgccg
      721 cagtcatggc cggcttcact cgcgaagtat tcgccaggac aacgacgctc gatctgcacc
      781 tattgatccg ccccgacacc gatttagacg gacacttccg cgcctggtgc actgacgagc
      841 aggagtggct gcggatcgag gggtggaact tctgcattca ggacgtaaac tctggagcgt
      901 ccgcatgatc gaggaggacg ctttgaagct caccaagctg cgcgctgaga ttcgcaaact
      961 caaccgcgag tccgacaagc tgatgcttga gactcgttgg tatccgatgg tcgtcaccac
     1021 ggcgctgttc gcagcggtcg cggcggtcat caagttgttc ggctgaatgc ctgaagtacc
     1081 agcgggagcg ctgccctagc gccacaccgg atgggctgcc tacggcccgg tggctggtga
     1141 gtaaatccta gcatcgcgca ggtctttgaa catcactgga aggcgcgttg cgctgtccga
     1201 aaattcccac aagccgcctc cgggcggctt ttttgttgcc tggagaaacc gcatggcctt
     1261 cgcctattac gaaaagggcg ccaccgtcct gctgctta
```

PCR Primers that amplify the *X. citri* 126 bp target sequence:
X. citri forward primer: 5′ TCCACTGCATCCCACATCTG
X. citri reverse primer: 5′ CAGGTGTACTGCGCTCTTCTTG

126 bp genomic sequence diagnostic for *X citri*, causal agent of citrus canker:
TCCACTGCAT CCCACATCTG GCACAAATCC CACGAAGGAG CGTGACATGA
CCAGGTATAA GGAAGCGCAC CACAGAAGAG TTCAAGCCGG CATGGGGAGG
CCGGCAAGAA GAGCGCAGTA CACCTG

Figure 4 *X. citri* PCR Assay Forward and Reverse Primers and Amplicon Sequence

3.1.1.4 Plant Infection Tests, Xanthomas DNA Extraction and Purification

Citrus plants (*Citrus sinensis* "Midnight" [Midnight Valencia orange], *Citrus sinensis* "Washington Naval" [Washington navel orange], *Citrus aurantiifolia* "Dwarf Bearss Seedless" [Bearss seedless lime], *Citrus limon* "Meyer" [Meyer lemon]) were obtained from Clifton's Nursery (Clifton's Flower and Garden Center, Porterville, California). Plants were grown and maintained, and plant infection tests were conducted in an environmental growth chamber with a 12-h day/night light cycle (ca. 300 µE/m^2/sec), 27°C daytime temperature, and 22°C nighttime temperature (Sherer Model 511-38 HLE). Overnight cultures of *Xanthomonas* were pelleted, washed twice with sterile tap water or sterile saline (0.85%), and resuspended to provide a titer of ca. 10^6 colony forming unit (CFU)/mL (titer confirmed by viable count). Citrus leaves were spray inoculated with the inoculum suspension using an aerosol chromatography sprayer (TLC sprayer, Analtech, Inc., Newark, Deleware) until the under surfaces of the leaves were thoroughly wetted. The sprayed leaves were enclosed in plastic bags and returned to the growth chamber; bags were removed after 24 h (Schaad 1988). Citrus-leaf tissues were sampled using a sharp, sterile cork borer (ca. 1-cm diameter, 0.8-cm^2 tissue area). Single leaf disks were transferred to sterile tubes (one leaf disk per tube), surface sterilized in a solution of 10% commercial bleach (Chlorox), 0.1% SDS for 5 min, rinsed with six changes of sterile water (5 mL, 2 min each rinse) and transferred to 2-mL ground-glass tissue grinders (Kontes Glass Cat. No. 885001-0002, obtained from Fisher Scientific). The disks were homogenized in 0.4-mL 1X PBS wash buffer (phosphate buffered saline, PharMingen, San Diego, California). The number of turns required to homogenize the leaf disks varied with the age of the leaf; grinding continued until all remnants of the intact leaves were homogenized. The desired product is a green slurry free from clumps of intact leaf tissue. The homogenized tissue suspensions were transferred to fresh sterile tubes and 1 mL of Qiagen buffer B1 (Qiagen Genomic-tip 20/G, Qiagen, Valencia, California) was added. Buffer B1 contains RNAse (200 µg/mL), proteinase K (1 mg/mL), and lysozyme (2 mg/mL) (all enzymes from Sigma, St. Louis, Missouri). *Xanthomonas* DNA isolation and purification followed the genomic DNA isolation protocol for bacteria supplied with the genomic DNA isolation kit (Genomic-tip 20/G; Qiagen, Valencia, California). The eluted DNA was precipitated overnight with 0.7 volumes room

temperature isopropanol, and glycogen (Sigma) was added as a carrier to 2 µg/µL. The precipitated DNA was collected by centrifugation, washed once with 70% ethanol, dried briefly, and resuspended in 50-µL sterile molecular biology grade water (Sigma) and stored at 4°C.

3.1.2 Results: Xanthomonas *axonopodis* pv. citri

3.1.2.1 RAPD Analysis

RAPD primers OPM-1, OPM-2, and OPM-12 produced diagnostic PCR fingerprints of *Xanthomonas axonopodis* pv. citri A (*X. citri*) strains (3210, 3213), which distinguished them from *X. campestris* strains and pathovars and *other Xanthomonas* species (data not shown). To determine whether any of the sequence contained within the *X. citri* RAPD amplicons was exclusive to the XacA genome, and thus could serve as a unique marker for a specific PCR assay, or was more widely distributed among various Xanthomads, gel purified, DIG-labeled XacA RAPD fragments were probed against dot blots of Xanthomonas DNA. The results of these assays (data not shown) indicated that the OPM-12-2 fragment could serve as the starting point for a diagnostic PCR assay for *X. citri*. While the OPM12-2 fragment was conserved in A and A* strains screened by Southern blot analysis, the OPM1-3 fragment was much less conserved and would not make a good (i.e., conserved) PCR target (see Figure 5). An approximately 3-kb *Pst*1 genomic DNA fragment that was hybridized to the OPM12-2 amplicon probe (1319 bp) and was conserved within XacA (A and A*) strains of world wide occurrence was not present in *X. axonopodis* pv aurantifollii pathotype B and C strains or X.a. pv. citrumelo isolates as demonstrated by Southern blot analysis of *Pst*I-digested genomic DNAs hybridized with probe OPM12-2 (not shown). Strain 165 from India had an RFLP as shown by the smaller (2.4 kb) band seen in Lane 4, Figure 5.

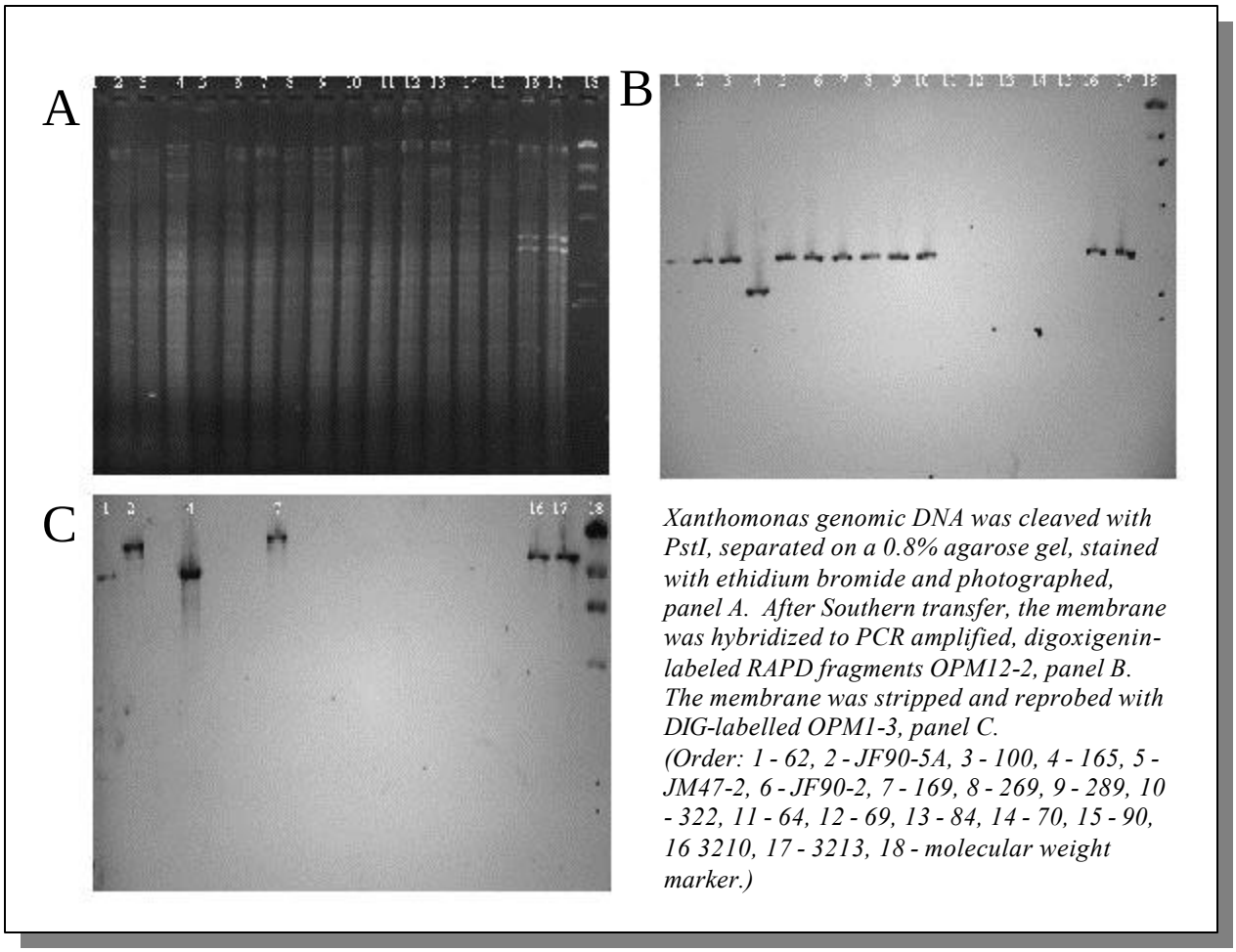


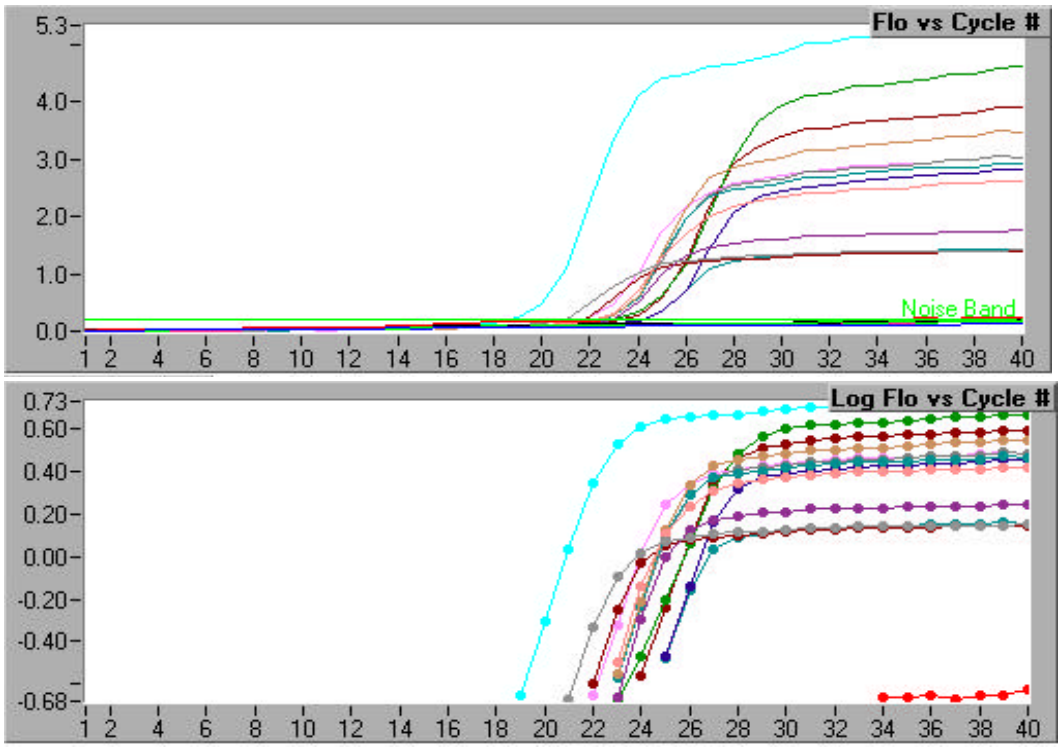
Figure 5. Southern Analysis of Xanthomonas Strains Probed with RAPD Fragments M12-2 and M1-3

The DNA sequence of the XacA strain 3210 M12-2 RAPD fragment is shown in Table 2. Querying this sequence against the non-redundant genetic databases with the National Center for Biotechnology Information (NCBI) BLAST (Altschul et al. 1990), revealed no significant homologies with sequences currently in the databases (the *X. citri* genome, currently being sequenced by a Brazilian consortium, will not be publicly available until sometime in late 2001). A 126-bp target amplicon, primer pairs, and TaqMan™ probe were identified by Primer Express, and are shown in Figure 4. The basic scheme for the TaqMan assay is shown in Figure 2. A variant of this assay using dual FRET probes was developed, and the scheme for this PCR assay is shown in Figure 3. Most analyses were performed with the TaqMan assay.

A TaqMan analysis of 19 *Xanthomonas citri* A and A* strains is shown in Figure 6. The differences in crossing-point threshold "Ct" (cycle number rise time) was due to minor differences in genomic DNA concentration between the different strains tested (see Figure 1 to review the effect of DNA target concentration upon the appearance of fluorescence). The upper panel plots relative fluorescence against cycle number (1–40) while the lower panel is a plot of log of fluorescence against cycle number—it is this graph that provides the Ct value. No reactions occurred with B and C strains tested. The sensitivity of the TaqMan assay with X. citri strain 62 genomic DNA is shown in Figure 7 (same data as shown in Figure 1). DNA concentrations ranged from 10–10^7 genomic equivalent copies per reaction. The sensitivity for detection was determined to be approximately 100 copies per reaction. The specificity of the assay was tested with a wide variety of *Xanthomonas* and other DNA samples (see Table 1). The results from one such heterologous control test are shown in Figure 8; only the Xc62 duplicate control reactions amplified positively. Three isolates from the current Florida canker epidemic were also tested and amplified positively, Figure 9. A mock citrus leaf infection experiment is shown in. This experiment was undertaken to determine what protocol provided optimum sensitivity (and ease of use) for recovering and detecting *X. citri* DNA directly from infected tissues.

The current limit of detection of X. citri from infected citrus leaves was approximately 103 targets per reaction. This is ten-fold less sensitive than the spike experiment, Figure 10, or the genomic sensitivity assay, Figure 7. This may be due to trapping of high molecular

weight DNA by the plant cell debris. Although in spiked (mock-infection) experiments we detected down to 102 copies per reaction. So the loss of sensitivity may be due to the limits to which we could completely homogenize the leaf disks, leaving some bacterial cells trapped within small clusters of plant cells and their DNA not extracted into the bulk solution. It was likely not due to plant-based inhibitors "killing" the PCR reaction since amplification and detection of 102 spiked cells was possible (see Figure 10).



X citri "A" strains amplify (increased fluorescence) (strains 62, 206, 251, 271, 273, 274, 275, 276, 278, 279, 280, 282, 283), while no reaction occurs with X.a, pv. aurantiifolii B (64, 69 84) and C (70) strains, and X.a. pv. citrumelo strains. Minor differences in DNA concentration affect the crossing threshold for detection. All X.citri A and A strains listed in Table 1 were tested and were positively detected by this assay (data not shown).*

Figure 6. TaqMan Analysis of 19 Xanthomonas Citri A and A*

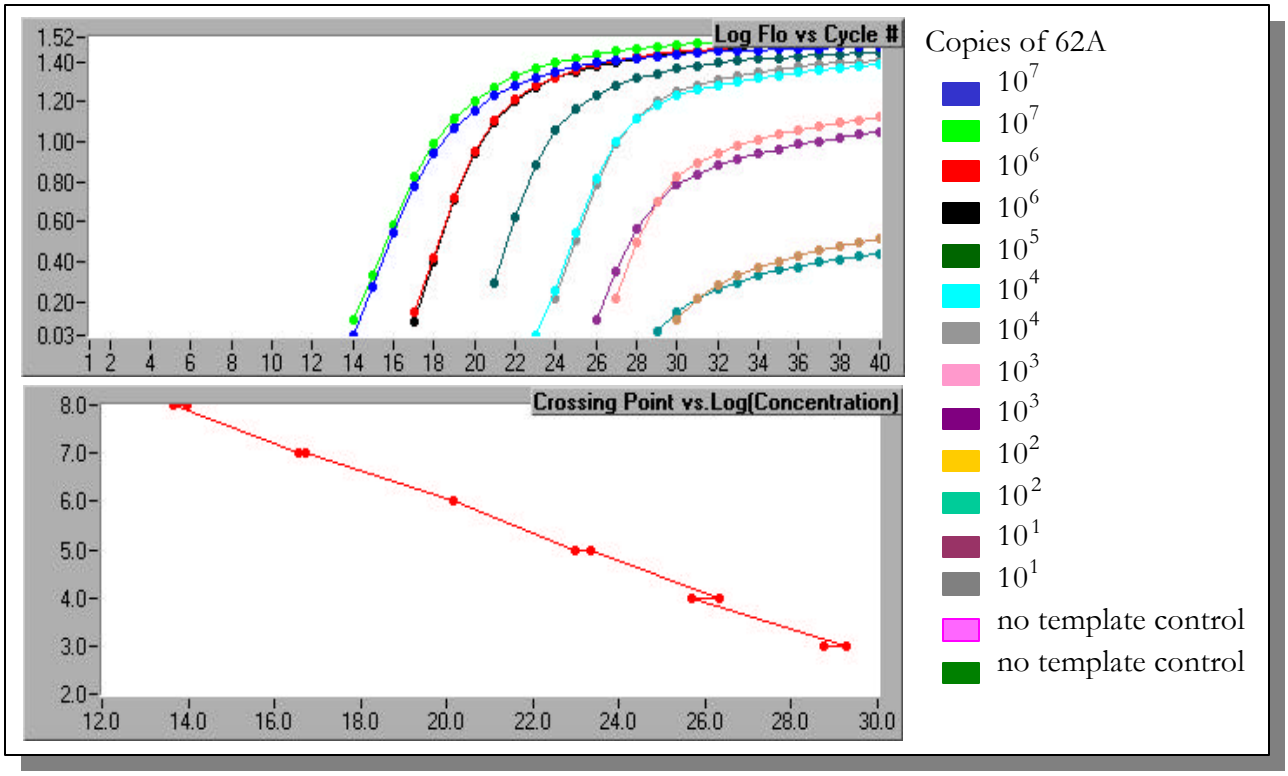
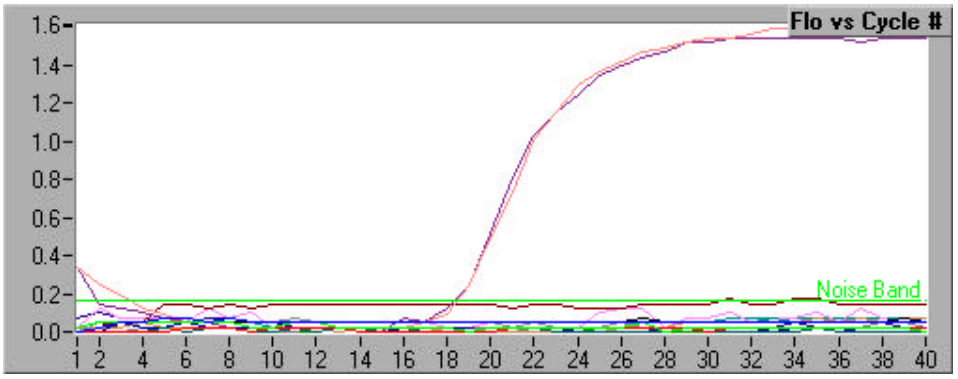
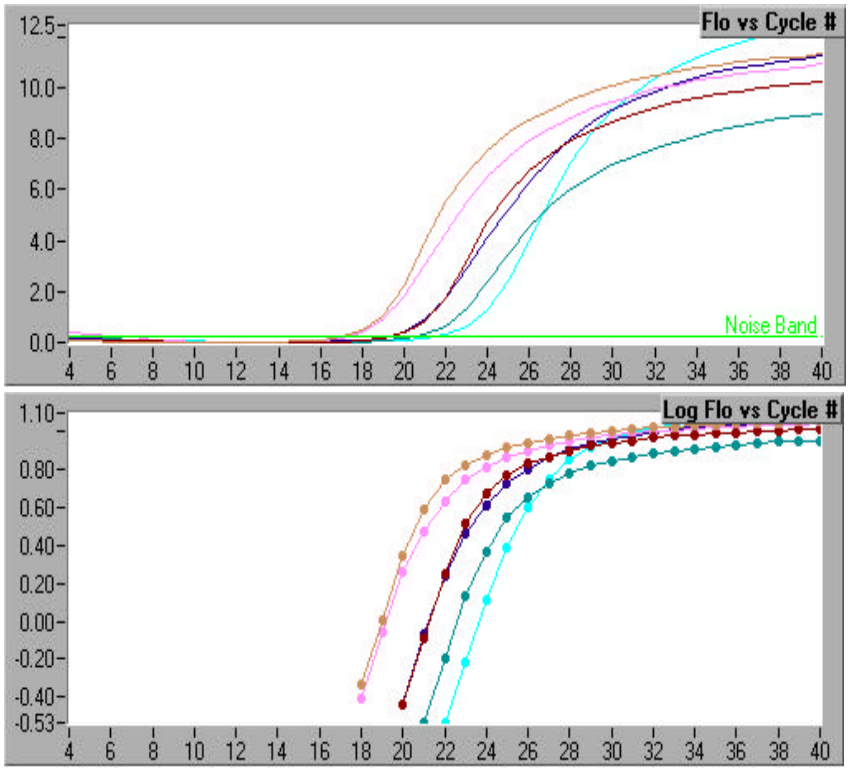


Figure 7. TaqMan Assay Sensitivity Test with X. Citri Strain 62 Genomic DNA. The assay has a detection limit of ca. 100 target copies per reaction with a chromosomal DNA sample.



The specificity of the TaqMan assay for X. citri strains was tested by running the assay against a wide variety of xanthomonads, citrus host DNA samples, and a number of other DNA samples listed in Table 1. Not all samples were tested in one analysis. There were no heterologous reactions detected. Duplicate samples of X.a. pv. citri strain 62 DNA served as a positive amplification control and were the only samples positively amplified.

Figure 8. Specificity of the TaqMan Assay for X. citri Strains



There was positive detection (increased fluorescence) with positive controls Xc62 , 3210, 3213, as well as the recent Florida outbreak strains X199-0112, XS97-0001-1, and XS97-012-1. There was no-reaction of the no template control, and strains 64, 69, 84, and ATCC 19312.

Figure 9. Detection of Current Florida *X. citri* Outbreak Isolates by TaqMan Assay

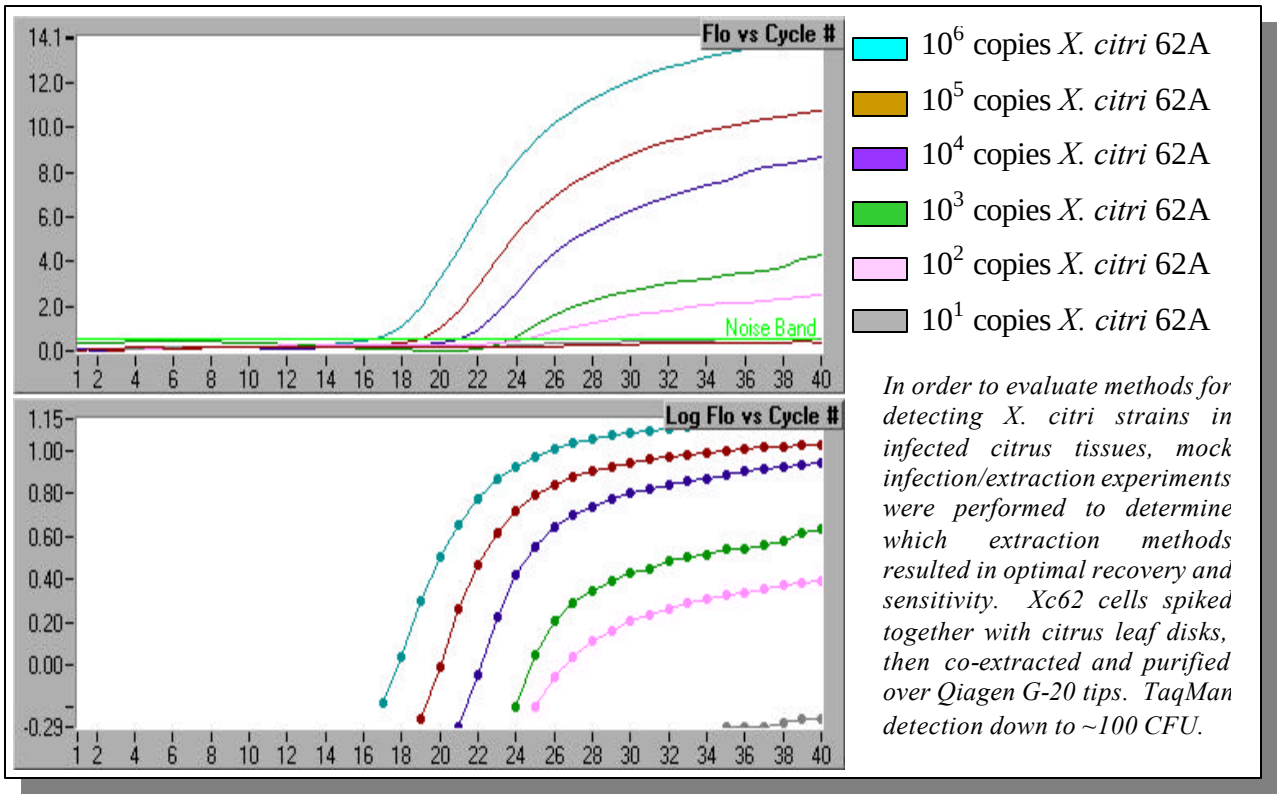
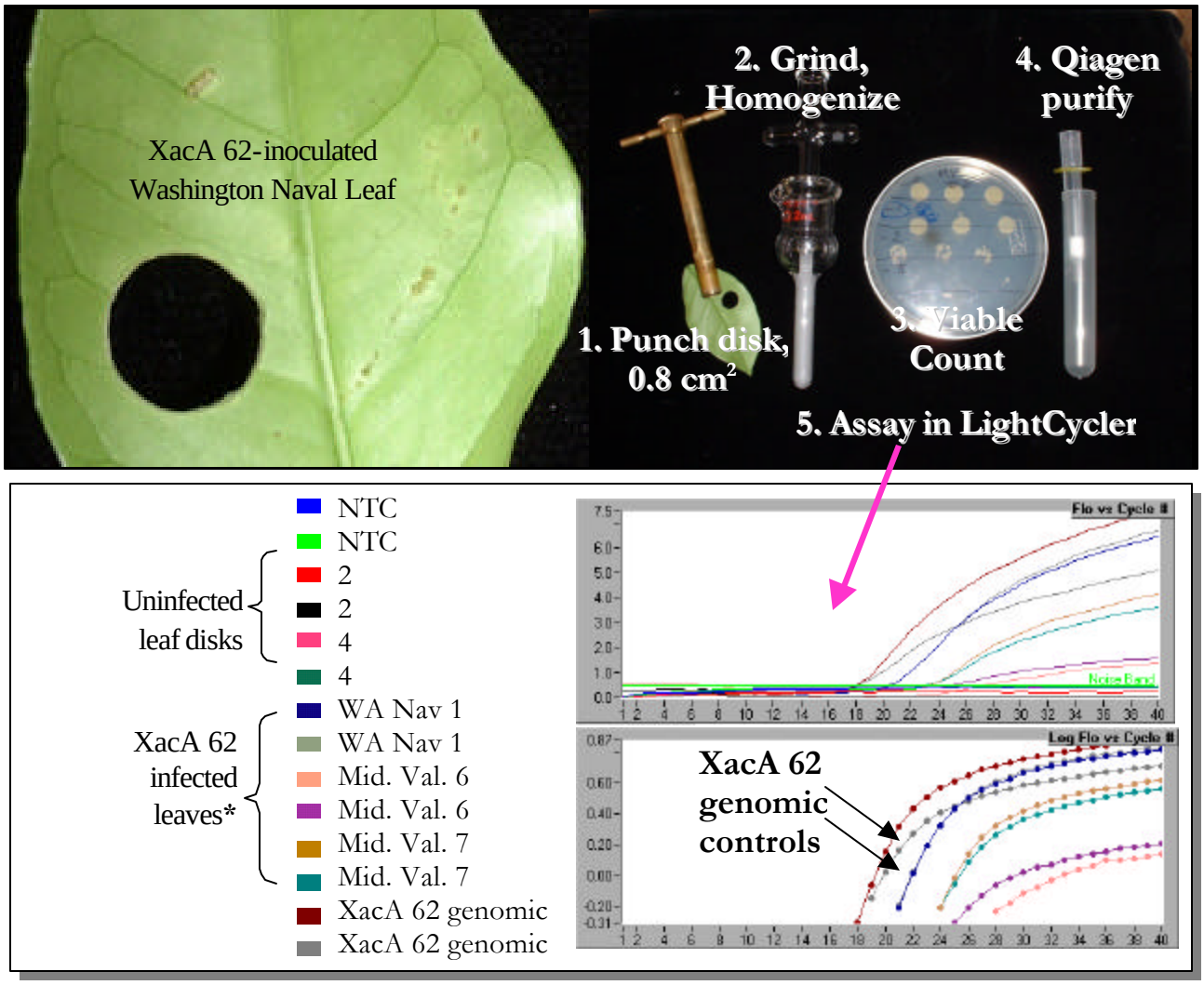


Figure 10. Xc 62 Cell-Spiked Citrus Leaf Disk Co-Extractions, TaqMan Assay.

Citrus leaves were spray inoculated with Xc62 as described previously, and 1-cm-diameter leaf disks were punched at different times post inoculation. The punched leaf disks were subjected to a two-stage isolation protocol. In Stage 1, the surface-sterilized leaf disks were homogenized in 450 µL PBS; a 50-µL aliquot was then removed to determine the viable titer. Dilutions of the leaf extract were made as deemed necessary and plated on TY-MOPS plates and incubated overnight at 30°C; the titer was expressed as per cm^2 leaf tissue. For Stage 2, the remaining 400 µL of leaf-disk homogenate was pelleted by centrifugation, the PBS buffer was decanted, and the pelleted leaf homogenate was resuspended in 1 mL of Quiagen B1 buffer and then subjected to Qiagen DNA extraction and purification following the Qiagen G-20 genomic tip protocol. The isopropanol-precipitated DNA was pelleted, rinsed with 70% ethanol, dried briefly, and resuspended in 50-µL molecular biology grade water (Sigma) and stored at 4°C. The extracted/recovered DNA was assayed in the LightCycler using the TaqMan assay. The results of one such infection experiment are shown in Figure 11.



* Washington naval leaf disk 1	(0.8 cm^2)	6.2 X 10^6 cfu/mL | 1.2 X 10^5 genomes/TL/LC rxn	
Midnight Valencia leaf disk 7	"	1.3 X 10^6 cfu/mL | 2.6 X 10^4 genomes/TL/LC rxn	
Midnight Valencia leaf disk 6	"	7.0 X 10^4 cfu/mL | 1.4 X 10^3 genomes/TL/LC rxn	

Figure 11. TaqMan Assay of X. Citri 62A Infected WA Naval & Valencia Orange

3.1.2.2 Discussion

A standard PCR assay for *X. axonopodis* pv. citri A (which also works on the A* strains (Verniere et al. 1998)) was previously reported (Hartung et al. 1993; Hartung et al. 1996) (Hartung assay). The Hartung assay targets a region on the virulence plasmid of *X. citri* strains, and therefore should only detect virulent isolates. X.c. isolates that have lost their virulence plasmid due to storage are not detected by this assay. We intended to adapt this assay to TaqMan, but experienced problems (no amplification) with some of the described primer sets. Since we had already made significant progress on the development of an assay based upon the M12-2 RAPD fragment, and to conserve research funds for other assays, we proceeded with the development of the PNNL assay rather than determine why the Hartung assay was not working for us. While plasmid copy number per cell can vary and potentially affect the level of sensitivity of detection (higher copy number, increased sensitivity), the chromosomal copy number should remain more constant. In addition, a chromosomal marker would likely not be lost upon storage of strains, as can occur with plasmids. The PNNL *X. citri* assay was based upon a chromosomal target that was present in all A and A* strains tested to date. This assay was optimized for the Idaho Technology LightCycler 32.

The *X. citri* TaqMan assay detected all A and A* strains in our collection and did not cross-react with other citrus-associated xanthomonads (i.e., X.a. pv aurantiifoli B and C strains, X.a. pv. citrumelo). When using purified genomic DNA, the assay had a detection limit of approximately 10^2 target copies per reaction. From Xc62-infected citrus leaves, the limit of detection was ca. 10^3 target copies per reaction. At the present time, we do not know the relevance (if any) of the target to *X. citri* biology, nor do we know its position and linkage within the *X. citri* genome. Once the complete nucleotide sequence of *X. citri* is published (perhaps in late 2001), it will be easier to determine what the target amplicon sequence is linked with in the genome. Currently, the target has no significant homology to any sequences in GenBank. Whether this unique target is necessary for some aspect of citrus host specificity could be postulated, but at this point that would be pure speculation. The assay of Hartung (Hartung et al. 1993; Hartung et al. 1996) is based upon a target linked with the virulence plasmid. Both assays should ultimately prove complementary in the

identification of *X. citri* A. Conversion of the Hartung standard assay to a fluorescence-based mode is currently ongoing (J. Hartung, Personal Communication).

Single tube, real-time fluorescent PCR assays provide a rapid and specific method for identifying pathogens. The *X. citri* TaqMan assay described here specifically detects and identifies the pathogen in a single assay with no subsequent offline analyses being required. The assay is capable of detecting *X. citri* in infected host tissues in less than 4 h of sample acquisition, with a detection limit of about 10^3 copies per reaction. For a pathogen of quarantine significance, like *X. axonopodis* pv. citri A, such rapid assays should provide for the prompt and accurate analyses and be of useful predictive value when determining zones of quarantine.

A manuscript describing the *X. citri* TaqMan assays is in preparation for submission to Applied and Environmental Microbiology. This work was presented at both the American Society for Microbiology (May 2000, Los Angeles, California) and the American Phytopathological Society (August 2000, New Orleans, Louisiana) annual meetings (Fritz and Kingsley 2000; Kingsley and Fritz 2000). Copies of these posters are provided as Appendix 6 and Appendix 7, respectively.

3.2 Tilletia Indica, *Causal Agent of Karnal Bunt of Wheat*

3.2.1 Background

Karnal bunt (KB) of wheat, caused by *Tilletia indica* Mitra, is a disease of significant economic importance to wheat growers as well as wheat exporters. *Tilletia indica* infects wheat, durum wheat and triticale. Currently, KB (which was first described in the village of Karnal, India, in 1930) does not occur within the United States. However, small outbreaks were discovered in 1996 in Arizona and California and in 1997 in Texas. Yield reductions in wheat due to Karnal bunt disease are slight; however, the pathogen is of concern because of its potential to have a severe economic impact. Many countries, including the United States, have a no tolerance policy towards Karnal bunt and refuse wheat shipments from quarantined countries or regions. The United States is the world's leading exporter of wheat, with an estimated annual value of $5 billion. Therefore, Karnal bunt poses a serious threat to international trade for the U.S. wheat industry (see (Frederick et al. 2000) for specific citations).

KB is a "disease" that has significance, not so much for its damage to the crop, but for its economic impact to wheat producers and exporters. In that regard, KB is similar to *X. citri,* a leaf spotting and fruit blemishing disease of citrus; both organisms are primarily of quarantine importance. KB teliospores could be employed in a manner analogous to food tampering. Teliopsores could surreptitiously be incorporated into a shipment of otherwise healthy wheat. Since there are several closely related *Tilletia* species that cause disease in small grains, a highly specific means of identifying the KB pathogen *Tilletia indica* is extremely important. Scientists at the USDA's Foreign Disease and Weed Science Laboratory at Ft. Detrick, Maryland, have produced a TaqMan assay that discriminates *T. indica* (wheat) from *T. walkeri* (rye) (Frederick et al. 2000). This assay was developed for the ABI 7700. We established a Material Transfer Agreement with the USDA FDWSL that allowed us access to this assay before its recent publication in Phytopathology (Frederick et al. 2000). Reid Frederick provided the primer, probe, and amplicon sequence information for the assay, and DNA for one (one each) isolate of *T. indica* and *T. walkeri*.

PNNL never established an APHIS permit for *Tilletia*; we simply wanted to adapt the ABI 7700 assay to the LC 32. Until the Frederick et al. article was published in the September 2000 issue of Phytopathology, USDA intellectual property representatives refused PNNL the ability to transfer specific probe, primer, and target amplicon sequence information to the FBI. With the publication of the article, the information covered under the material transfer agreement (MTA) is now in the public domain. Some of the following information was previously reported in the 4Q99 Quarterly Report.

3.2.2 Results: TaqMan Assay for Karnal Bunt

Purified genomic DNA from *Tilletia indica* (wheat isolate, WL1562) and *Telletia walkeri* (ryegrass isolate, 210G) was obtained from Dr. Reid Frederick (USDA, Ft. Detrick). We had the following olgionucleotides synthesized commercially:

Tin3 - 5' CAATGTTGGCGTGGCGGCGC (*T. indica*-specific forward primer);

Tin11 - 5'-TAATGTTGGCGTGGCGGCAT (*T. walkeri*-specific forward primer);

Tin10 - 5'-GAGGAACTTGAGGCGGAGCT (common reverse primer); and

KB TaqMan probe 5' 6-FAM-ATTCCCGGTTCGGCGTCACT-TAMRA.

A series of experiments was undertaken to optimize the TaqMan conditions for use in the LightCycler (primer and template concentrations, Mg^{2+} concentration, optimal TaqMan probe concentration; data not shown).

Positive amplification and detection results were obtained using the following reaction conditions for each sample: 0.5 µM of each PCR primer, 0.05 µM KB TaqMan probe, 0.2 mM dNTP, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mg/mL BSA, 8 ng of genomic DNA, and 1 unit of Qiagen Taq polymerase mixed with Taqstart antibody (Clonetech Laboratories, Inc., Palo Alto, California). The following cycling conditions resulted in a successful LC32 TaqMan assay: denature at 94°C, 0 sec, 20°/sec ramp rate; 56° anneal 0 sec, 20°/sec ramp rate; 62° extend, 20 sec, 2°/sec ramp rate (40 cycles). Acquisition of 6-FAM fluorescence occurred at the end of each extension cycle. The specifics of the KB assay on the LC32 are summarized Figure 12. Fluorescence increase was observed with *T. indica* WL1562 DNA template only in the presence of the 3/10 primer set; alternatively, fluorescence increase was observed for *T. walkeri* 210G DNA only in the presence of the 11/10 primer set (see Figure 13).

The cycling conditions for the LightCycler were significantly different than those reported for the Perkin-Elmer 7700 instrument. The assay in the 7700 is a two temperature PCR of 34 cycles with 95° denature for 15 sec followed by an anneal/extension at 60° for 1 min. The other significant difference was the amount of probe required for the assay. Fluorescence detection in the 7700 required a 0.5 µM probe compared to a 0.05 µM probe for detection in the LightCycler.

KARNAL BUNT TAQMAN ASSAY*

Adaptation to the LightCycler32

KB TaqMan Probe
5' 6-**FAM**-ATTCCCGGTTCGGCGTCACT – **TAMRA**

Tin3 –(*T. indica*-specific) **Forward primer**
5'-CAATGTTGGCGTGGCGGCGC

Tin11 - (*T. walkeri*-specific) **Forward primer**
5'-TAATGTTGGCGTGGCGGCAT

Tin10 - Reverse Primer (common)
5'-GAGGAACTTGAGGCGGAGCT

Reaction Conditions
0.5 µM of each PCR primer
0.05 µM TaqMan probe
0.2 mM dNTP
5 mM $MgCl_2$
10 mM Tris-HCl (pH 8.3)
50 mM KCl
0.25 mg/ml BSA
8 ng of genomic DNA
1 unit of Qiagen Taq polymerase mixed with Taqstart antibody

LightCycler32 Conditions
Template detection occurs within 40 cycles of:
Denature at 94° C, 0 sec, 20°/sec ramp rate
Anneal at 56° C, 0 sec, 20°/sec ramp rate
Extend at 62 ° C, 20 sec, 2°/sec ramp rate

Acquire 6-FAM fluorescence at the end of each extension cycle.

Figure 12. KB Taqman Assay Protocol

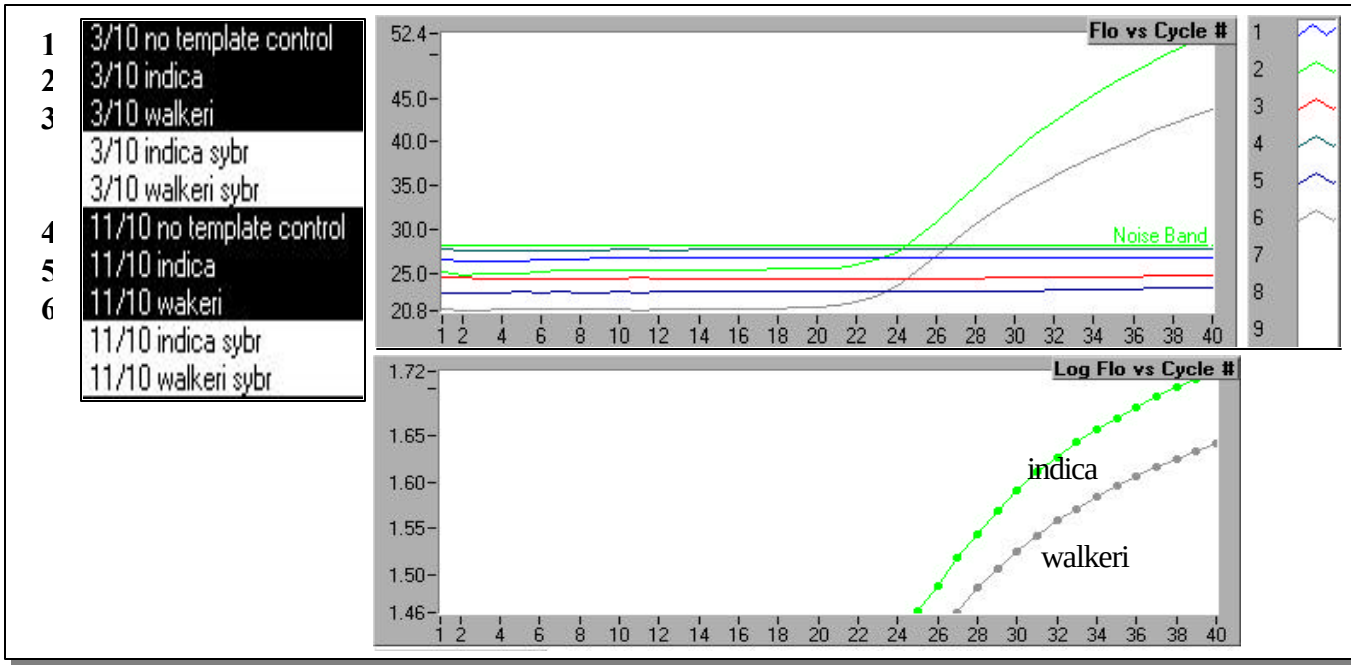


Figure 13. KB TaqMan assay on the LightCycler

3.3 Fusarium graminearum/Gibberella zeae

3.3.1 Background

Mycotoxin contamination of food and feed is one of the most important agricultural and health problems worldwide. *Fusarium graminearum* (teleomorph: *Gibberella zeae*) (Fg/ Gz) causes head scab, or head blight of wheat, barley, and oats, and foot and crown rot of corn (Trail 2000). Scab is an economically devastating plant disease because it causes significant reduction in seed yields and quality, but also because infested seeds are often contaminated with tricothecene and estrogenic mycotoxins. These mycotoxins pose a serious threat to animal health and food safety. The major impact of Fg is the production of the tricothecene mycotoxin deoxynivalenol (DON) upon infection of developing grain. DON is a potent inhibitor of protein synthesis that affects humans and animals that consume the contaminated grain (or grain products made from DON-contaminated grain). The polyketide zealerone is a second mycotoxin that is produced by G. zeae and has estrogenic activity in mammals. During the past decade, scab or *Fusarium* head blight of wheat and barley has reached epidemic proportions in North America, as well as elsewhere in the world. Between 1991 and 1996, because of decreased yields and price discounts as a result of poor seed quality, ca. 3 billion dollars has been lost in the United States because the winter and spring wheat and barley crops were contaminated by DON (O'Donnell et al. 2000; Trail 2000).

Complicating the picture, scab can be caused by four species of *Fusarium*: *F. graminearum, F. culmorum, F. avenaceum* and *F. crookwellense*. Only Fg and *F. culmorum* produce DON. In North America and continental Europe, Fg is the most important scab-causing fungus (Trail 2000). The recent phylogenetic/phylogeographic analysis conducted by O'Donnell et al. (O'Donnell et al. 2000) revealed seven biogeographically distinct lineages within the Fg clade. This result indicates a long evolutionary history of isolation. It also means that we will need to expand our *Fusarium* isolate collection (or obtain DNA samples from H. Kistler, K. O'Donnell) to test any potential Fg/Gz TaqMan assay.

From sources in the literature, four primer pairs were reported to be useful for identifying *F. graminearum* (Figure 14). We found that these assays also amplified the teleomorph G.z; to date, we have not identified any specific PCR assays in the literature for *Gibberella zeae*.

As is described later in the Results section, our initial attempts at a TaqMan assay appeared initially successful until we tested the assay against other *Fusarium* species and strains and ran the assay for more than 30 cycles. Although the PCR assays described below were published as being discriminative for Fg, and our initial screens of heterologous fusaria using the published primer sets and protocols (standard PCR) were negative, our initial efforts to convert the GaoA to a fluorescent PCR was not successful because of a lack of specificity of the TaqMan assay in the LC32.

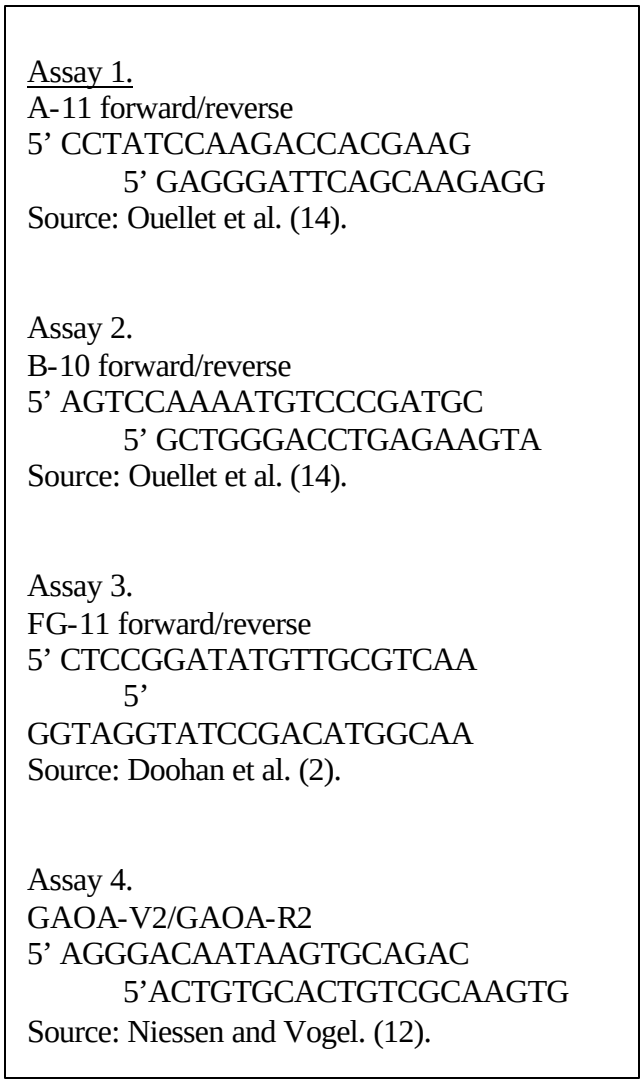


Figure 14. Published PCR Assays for Fusarium Graminearum

Upon receipt of USDA-APHIS permit approval for receiving, culturing, and working with Fg/Gz isolates in the laboratory for DNA isolation, we obtained a collection of *F. graminearum* and *Gibberella zeae* from ATCC (Table 2). We are currently not

Table 3. Fusarium Strain Collection

ATCC Number	Organism	Isolation
15624	Fusarium graminearum Schwabe	Corn stalk, Canada
20334	Fusarium venenatum Nirenberg	Soil, United Kingdom
26557	Fusarium graminearum Schwabe	Zea mays moldy grain, USA
34909	Fusarium graminearum Schwabe	Millet, Hungary
34912	Fusarium graminearum Schwabe	Millet Hungary
36882	Fusarium graminearum Schwabe	Barley, Finland
36884	Fusarium graminearum Schwabe	Barley, Finland
36885	Fusarium graminearum Schwabe	Wheat, Finland
44418	Fusarium graminearum Schwabe	Winter wheat seed, Hungary
46779	Fusarium graminearum Schwabe	Corn
56091	Fusarium graminearum Schwabe	Rice stem, Italy
56092	Fusarium graminearum Schwabe	Soft wheat stem, Italy
56093	Fusarium graminearum Schwabe	Maize ear, Italy
58667	Fusarium graminearum Schwabe	Fodder, Finland
60289	Fusarium graminearum Schwabe	Maize grain, New Zealand
60309	Fusarium graminearum Schwabe	White winter wheat, Canada
60880	Fusarium graminearum Schwabe	Maize grain, New Zealand
60881	Fusarium graminearum Schwabe	Maize grain, New Zealand
16106	Gibberella zeae (Schweinitz) Petch	Corn stalk, New York
20028	Gibberella zeae (Schweinitz) Petch	Corn
20271	Gibberella zeae (Schweinitz) Petch	Moldy corn
20273	Gibberella zeae (Schweinitz) Petch	?
24688	Gibberella zeae (Schweinitz) Petch	Corn Minnesota, single ascospore isolate
24689	Gibberella zeae (Schweinitz) Petch	Corn Minnesota
28106	Gibberella zeae (Schweinitz) Petch	Japan
36015	Gibberella zeae (Schweinitz) Petch	Corn Indiana
36016	Gibberella zeae (Schweinitz) Petch	Corn Indiana
24373	Fusarium graminearum Schwabe	Zea mays, South Africa
20329	Fusarium graminearum Schwabe	United Kingdom

approved for plant infection tests with these Fg/Gz isolates, which could be obtained by requesting, from APHIS, an amendment to the current permit. Work commenced upon assay development in January 2000. The Fg/Gz isolates were cultured and their DNA isolated and purified.

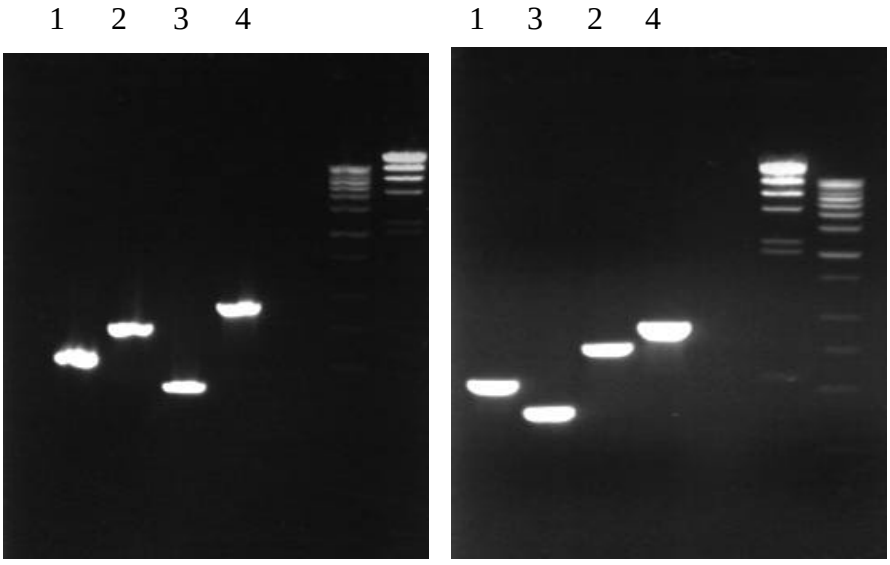
Primers A-11, Fg-11, B-10, and GaoA, published as diagnostic for *Fusarium graminearum*, tested positive against our *Fg/Gz* collection in standard PCR tests following the author's protocols. These primers were also tested against a group of 10 other *Fusarium* strains (Table 4) with *negative* results in these standard PCR tests.

Table 4. PNNL Fusarium Strains

PNNL #	Strain, ATCC #	Host
1357	*Fusarium lateritium* 38557	mulberry
1338	*Fusarium oxysporum* 62125	Spinach seed embryos
1339	*Fusarium moniliforme* 60846	corn
1362	*Fusarium solani* fsp *phaseoli* 60860	bean
1702	*Fusarium buharicum* 24135	*Hibuscus cannabinus*
1705	*Fusarium coccophelium* 24365	coffee
1706	*Fusarium compactum* 15618	Peanut
1711	*Fusarium decomcellulare* 16562	*Kola acuminata*
1727	*Fusarium moniliform intermedium* 48846	*Phoenix dactylefera* (Iraq)
1734	*Fusarium acuminitum* 32965	*Poa pretensis*

Our initial goal was to convert each of these assays into a TaqMan assay for comparative purposes and then select the one or two that appeared most effective. However, due to time considerations and the desire to deliver a working assay before the project was completed, we decided to focus on the GoaA assay of (Niessen and Vogel 1997) since it was a defined target. We selected *F. graminearum* strain ATCC 26557 (corn) and *G. zeae* strain ATCC 16106 (corn) as our sources for amplicon target generation for cloning and sequence analysis; both strains were isolated from corn in the United States. DNA from *F. graminearum* 26557 and *F. zeae* 16106 was amplified with the primer sets described in Figure 14, and the amplicons ligated into the *E. coli* vector TOPO II then transformed into *E. coli.* Clones containing the appropriately sized inserts were selected and used for DNA sequence analysis for TaqMan assay development.

Fg ATCC 26557 and Gz ATCC 16106 were amplified by PFU Turbo Taq polymerase (Stratagene) (PFU used since the products were being cloned into the TOPO vector system) with primers A-11, Fg-11, B-10, and GaoA. An aliquot of the amplification was separated on a 1% agarose gel to determine purity, concentration, and molecular weight using GelExpert software (GelExpert 97 v2.0, NucleoTech Corp., San Carlos, California). (GelExpert software comes with the NucleoVision gel documentation system (NucleoTech Corp., San Carlos, California). The software calculates molecular weights based upon DNA marker lanes and provides DNA concentration information based upon densitometry with DNA reference standards. The system will also count colonies on plates and filters and quantitate DNA blot, slot blot, and ELISA analyses.) The remainder of the sample was reserved for cloning into the



Lane	Primers	*F. gram*	*G. zeae*
1	A-11	580	500
2	B-10	771	751
3	Fg-11	416	376
4	gaoA	915	889

One clone was selected from each ligation for large scale DNA purification. Each clone was amplified with insert specific primers as final verification of appropriate insert. The resulting amplicons were separated on a 1% agarose gel, and their molecular weights determined using GelExpert software.

Figure 15. Molecular Weight Comparison of Inserts Amplified from F. Graminearum and G. Zeae

TOPO II vector (Invitrogen) and transformed into E. coli. The relative size differences between the Fg and Gz products were an artifact caused by running the samples of two separate gels. In fact, there was actually little difference in insert length between the *F. graminearum* and *G. zeae* amplification products (verified by the sequence analysis).

The preliminary sequence analysis of inserts A-11 and Fg-11 revealed possible open reading frames. DNA sequence analysis also revealed considerable homology between *F. graminearum* and *G. zeae.* However, a BLAST search of the nucleotide sequences against the NCBI database revealed no sequence homologies to known sequences. Due to time constraints, we chose to focuse only on developing one of the assays. The evidence provided Niessen and Vogel (Niessen and Vogel 1997) appeared to be the most convincing in terms of specificity of their assay. For this reason and also due to the limited amount of time remaining before the termination of the project, we decided to produce a TaqMan assay based upon the GaoA target. A TaqMan probe and primer set was designed from the GaoA sequence using Primer Express (Applied Biosystems). The LightCycler program consisted of: **melt** - 94°C, 0 sec., 20°C/sec ramp; **anneal** - 56°C, 0 sec., 20°C/sec ramp; **extend** - 66°C, 10 sec., 2°C/sec ramp, with a single fluorescence acquisition (**acquire**) at the end of the extend cycle. The amplicon length in this assay is 71 bp. The forward and reverse primers were ordered from Genosys, and the GoaA TaqMan probe was purchased from Applied Biosystems. The assay protocol is summarized in Figure 17.

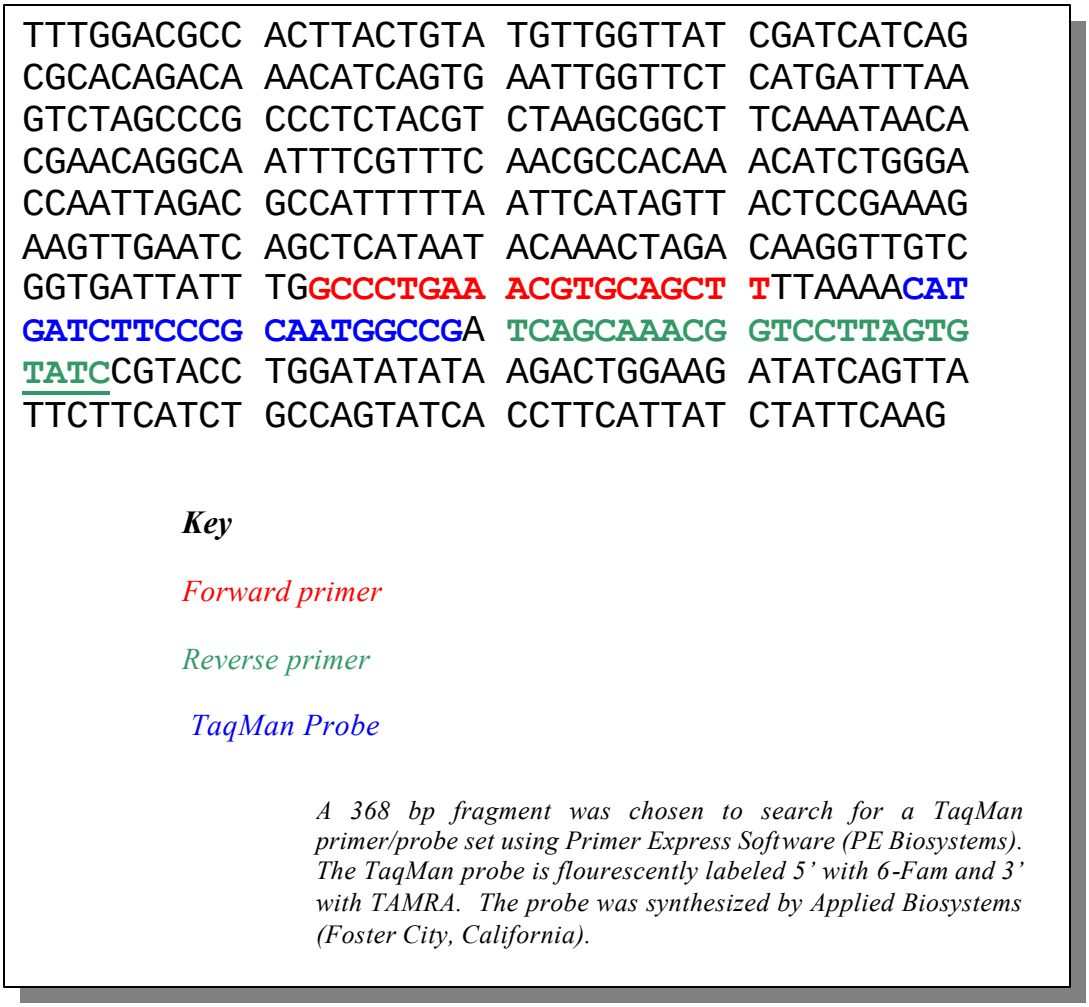


Figure 16. TaqMan Probe and Primer Set for GaoA

***Fusarium graminearum* TaqMan Assay**

TaqMan Probe
6-**FAM**-CATGATCTTCCCGCAATGGCCG-**TAMRA**
Forward Primer
5' GCCCTGAAACGTGCAGCTT
Reverse Primer
5' CTATGTGATTCCTGGCAAACGCACT

Reaction Conditions
0.5 mM of *Fg* forward and reverse primers
0.05 mM TaqMan probe
0.2 mM dNTPs
5 mM $MgCl_2$
10 mM Tris-HCl (pH 8.3)
50 mM KCl
0.25 mg/ml BSA
0.5 – 1 unit of Taq DNA polymerase and template DNA
(5'-3' exo+ Taq)*

LightCycler Conditions
Template detection occurs within 40 cycles of:
denature at 94°C for 0 sec, ramp rate of 20°/sec
anneal at 56°C for 0 sec, ramp rate of 20°/sec
extension at 66°C for 10 sec, ramp rate 2°/sec

(Acquisition of the fluorescent signal occurs at the end of each extension cycle excite/read **FAM** fluorescence FRET-blocked by TAMRA until cleaved by Taq exonuclease activity)
*(Qiagen Taq DNA polymerase)

Figure 17. *Fusarium graminearum* GaoA-Based TaqMan Assay Protocol

3.3.2 Results and Discussion: Fusarium graminearum

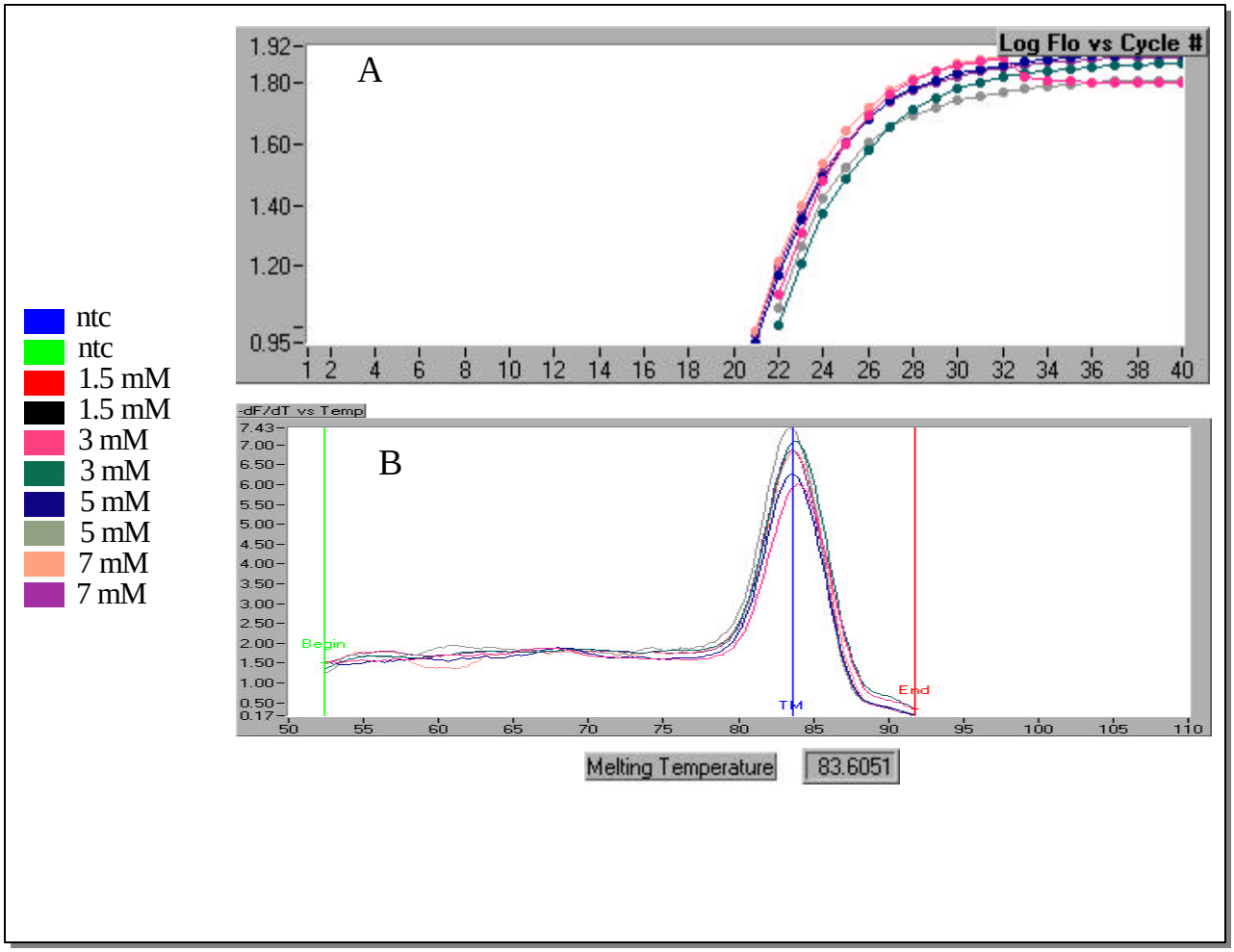
To optimize the assay parameters, the optimum concentrations of Mg^{2+} (Figure 18) and TaqMan probe (Figure 19) were determined in a series of LightCycler experiments. Four concentrations of Mg^{2+} (1.5, 3, 5, and 7 mM) were used to amplify F. graminearum 26557. Amplification was monitored with SYBR green fluorescence. No amplification occurred with the 1.5-mM reactions. Five mM Mg^{2+} was selected for TaqMan assay development. Fg TaqMan probe concentrations of 50 and 100 nM were used to monitor the amplification of F. gram. 26557 and G. zeae 16106. A concentration of 100 nM extends the linear portion of the curve without increasing background, but 50 nM was employed for general assay use.

Our initial screens with heterologous *Fusarium* strains (in the Techne Genius thermocycler, standard PCR, gel-based product analysis) did not demonstrate any cross-reactions. This result gave us the impression that these published assays were specific for Fg. In the future, all heterologous reaction tests will be conducted for at least 40 cycles of amplification. It is apparent from the TaqMan assay results illustrated in the following figures that the cross-reactions occur (and are detectable) after 30 cycles. However, these results may be due to problems with this specific TaqMan probe and primer combination.

To ensure that the DNA samples from the heterologous Fusarium test (Figure 21) were not contaminated with F. gram/G. zeae DNA, other stored DNA preparations were accessed. These had never been opened or sampled during this research project. The purity of the TaqMan master mix was also tested by running a no template, negative control (ntc) in quadruplicate. The results of this experiment (Figure 21) are similar to the previous experiment. Amplification of the alternate Fusarium strains occurred at later times. This late amplification is not due to DNA concentration, as high and low concentrations of DNA yielded the same result (i.e., higher concentrations of alternate strains did not cause the heterologous amplification to occur at an earlier cycle). The no-template controls did not amplify, so contamination of the reagents with Fg/Gz DNA was unlikely. The non-specific amplifications occur only in the presence of *Fusarium* DNA templates, as the ntc controls in experiments shown in Figures 21 and 22 were blank. Another experiment needs to be performed to determine if these false amplifications are specific to Fusarium DNA or whether bacterial or other DNA samples (Table 1) would result in non-specific amplification

with this set of GoaA primers and TaqMan probe. The problem may lie with this particular primer set and TaqMan probe.

Amplification of the alternate *Fusarium* species suggests that the GaoA-derived TaqMan probe and primers are not specific to *F. gram/G. zeae* as had been implied in the references for the standard PCR assays. These amplifications occurred late in the reaction program. The fact that they seem to be DNA-template dependent (no amplifications in ntc controls), yet independent of heterologous *Fusarium* template concentration, indicates that a template is important for initiating the reaction, but that an additional template does not cause the false amplifications to occur earlier. We speculate that this might be a problem of the probe annealing to an alternate sequence, thereby becoming a target for the 5′ exonuclease activity of the polymerase. This hypothesis, as yet untested, will be checked by determining amplicon melting temperature in the presence of probe using the DNA intercalating dye SYBR green. However, such a phenomenon would most likely be template-concentration responsive. Modifications of the reaction chemistry (lower Mg^{2+} concentration) should also be evaluated for effects upon reaction specificity. Also the use of TaqStart antibody (Ab Peptides) or HotStarTaq (Qiagen) to provide a hot-start PCR reaction, which is known to decrease primer dimmer formation and nonspecific priming, might alleviate the problem as well. Several simple, but important, reaction tests need to be performed before ruling out the current GoaA-based TaqMan assay for Fusarium graminearum in favor of a new primer and probe combination, or basing the assay on an entirely different target.



Four concentrations of Mg $^{2+}$ (1.5, 3, 5 and 7 mM) were used to amplify F. graminearum 26557. Amplification was monitored with SYBR green fluorescence (panel A). Differences in cycle thresholds between Mg $^{2+}$ concentrations 3 and 7 mM were not significant (panel A). Following the PCR reaction, melting temperature of the amplicon was measured at 83.6^{o} C using the LightCycler melt program (panel B).

Figure 18 Optimization of Mg 2^{+} Concentration for TaqMan Primers in F. Gram./G Zeae Assay

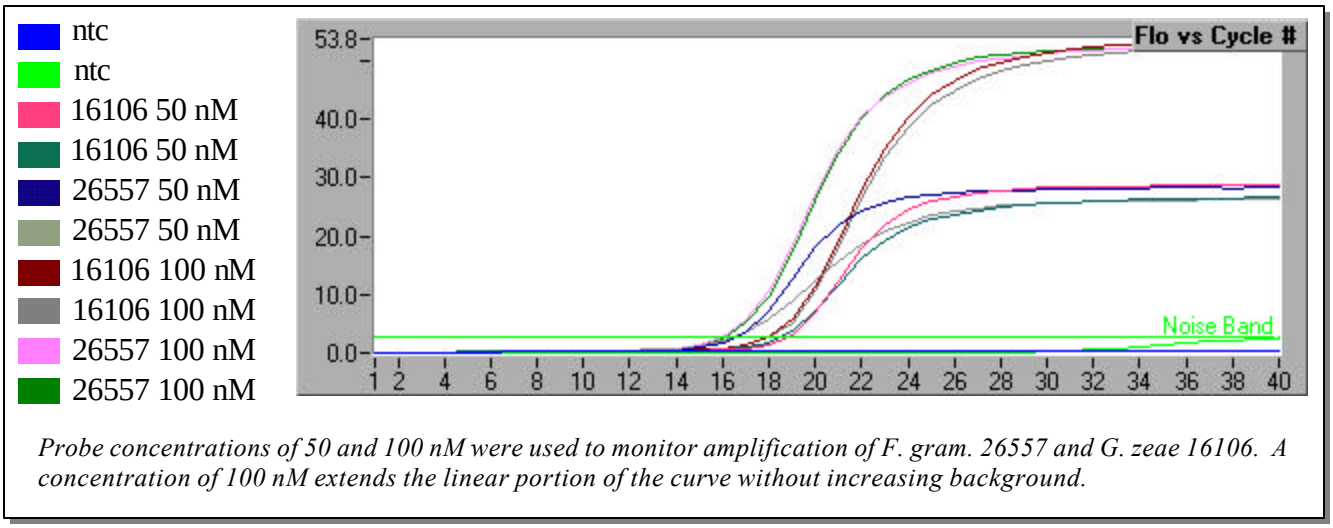
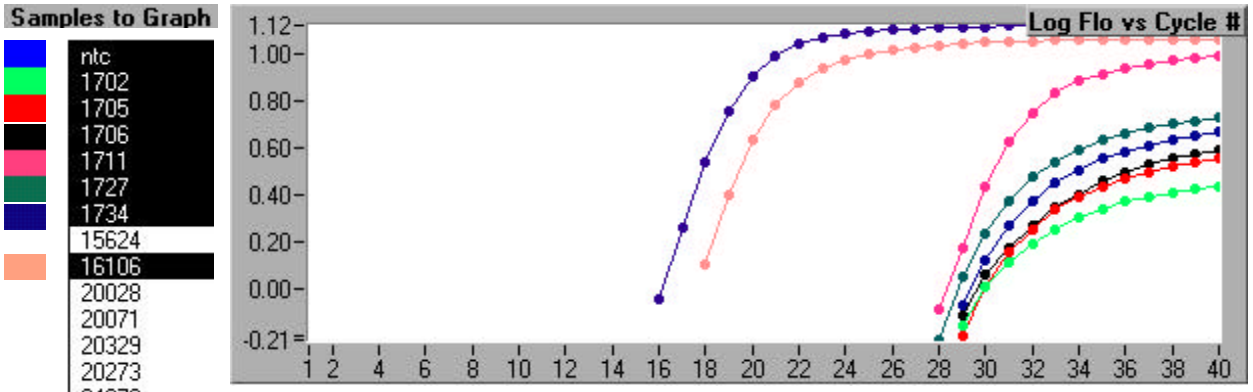


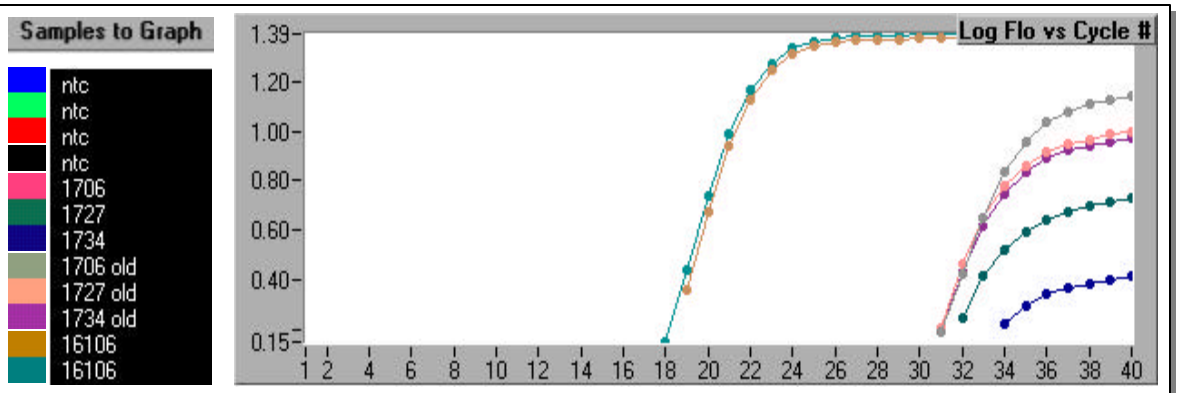
Figure 19 Optimization of TaqMan Probe Concentration for F. Gram./G. Zeae TaqMan Assay



Various alternate strains were used (see key) to test the specificity of the F. gram/G. zeae TaqMan assay. For clarity, only the black highlighted samples in the legend are plotted. Cycle thresholds for the F.gram/G. zeae strains were ~cycles 16-22 (not all data shown). Alternate strains also amplified with cycle thresholds at 27-29.

PNNL Strain number	ATCC number	Species name
1702	24135	*F. buharicum*
1705	24365	*F. coccophelium*
1706	15618	*F. compactum*
1711	16562	*F. decumcellulare*
1727	48846	*F. moniliform intemedium*
1734	32965	*F. acuminitum*

Figure 20 F. Gram/G. Zeae TaqMan Assay Using Heterologous Strains/Species of Fusarium



To ensure that the DNA samples from the heterologous Fusarium test Figure 21) were not contaminated with F. gram/G. zeae DNA, other stored DNA preparations were accessed. These had never been opened or sampled during this research project. The purity of the TaqMan master mix was also tested by running a no-template negative control (ntc) in quadruplicate. The results of this experiment are similar to the previous experiment. Amplification of the alternate Fusarium strains occurred at later times. This late amplification is not due to DNA concentration, as high and low concentrations of DNA yielded the same result (i.e., higher concentrations of alternate strains did not cause the heterologous amplification to occur at an earlier cycle).

Figure 21 Retest of Alternate Fusarium Strains

4.0 Conclusions

This project:

- Produced a Technical Requirements Document that outlined the status of PCR assays for phytopathogens
- Developed a network of contacts and experts in the field of phytopathology
- Purchased an Idaho Technology LC32 so assays developed at PNNL would be directly compatible, without modification, with the HMRU instrument
- Developed a TaqMan assay for the bacterium *X. citri*, causal agent of citrus canker
- A manuscript, describing the *X. citri* TaqMan assays is in preparation for submission to Applied and Environmental Microbiology.
- The *X. citri* assay work was presented at both the American Society for Microbiology (May 2000, Los Angeles, California) and the American Phytopathological Society (August 2000, New Orleans, Louisiana) annual meetings (Fritz and Kingsley 2000; Kingsley and Fritz 2000). Copies of these posters are provided as Appendix 6 and Appendix 7, respectively.
- *X. citri* assay primers and TaqMan probe deposited in GenBank
- Adapted an assay for Karnal bunt of wheat to the LightCycler
- A manuscript detailing the comparison of the TaqMan assay for Karnal bunt performed in the ABI 7700 and the LC32, in collaboration with Reid Frederick of the USDA-FDWSL, will be prepared for the American Phytopathological Society web-based journal Plant Health Progress.
- Initiated development of a TaqMan assay for the fungus *Fusarium graminearum*

PNNL is well suited to conduct molecular phytopathology studies. In addition to well-equipped molecular biology/microbiology laboratories we are have approximately 4800 ft^2 of controlled-access greenhouse space and approximately 108 ft^2 (total) of restricted-access environmental growth chamber space. We are able to maintain pathogen-infected plants under a variety of containment conditions that meet strict APHIS permitting requirements.

Nucleic acid-based methods for the detection and identification of organisms provide great resolving power and sensitivity. They also provide a means for detecting, via

polymerase chain reaction DNA amplification, trace amounts of dead biologicals that could be present in forensic evidence. PCR-based methods such as TaqMan, which includes the means to specifically identify (and quantify) the desired target amplicon based on fluorescence, alleviates the need to transfer samples and perform gel-based analyses. DNA amplification and target detection can occur simultaneously and only a single tube needs to be loaded; there are no subsequent transfers of samples required.

Phytopathogen diagnostics would greatly benefit from an increased use of fluorescent PCR methods. Currently, there are few phytopathology laboratories equipped with fluorescent PCR thermocyclers, although their adoption is increasing due to the reduced cost associated with newer machines coming to market. Consequently, there remains a limited number of assays available for the majority of pathogens that (i) cause significant damage to crops (ii) are a problem due to export/import quarantines or (iii) might pose a significant risk for crop-targeted biological terrorism. Therefore a continued effort is required to develop TaqMan diagnostics for significant crop pathogens.

Future efforts would entail completion of the Fg/Gz assay initiated during the FY98-00 project**.** These topics are covered in more detail in a proposal for follow on research and development efforts, but a proposed set of year FY01-02 tasks from the follow on proposal is reproduced below.

Assays/Tasks for year '01-'02:

- Completion of *Fusarium graminearum/Gibberella zeae* TaqMan assay (started in FY00) consult with Korby Kistler and Kerry O'Donnell (both USDA) regarding *Fusarium* phylogenetics and putative target amplicon choices

- Conduct literature review and analysis of population structure of Puccinia – review what targets might be useful for a TaqMan assay, determine what type of TaqMan assays could be developed with the current molecular genetic knowledge of the genus vs. the need for extensive phylogenetic analysis. Rolland Line (WSU) and the researchers at the USDA Cereal disease lab can provide significant input into this analysis

- Adapt Sorghum ergot assay to the LC32, once development is finalized by Paul Tooley/FDWSL

- Collaborate with Reid Frederick/FDWSL on Soybean Rust TaqMan assay adaptation to the LC32

- Commence development of a TaqMan assay for bacterial blight of cotton.

5.0 LITERATURE CITED

Altschul, S. F., W. Gish, W. Miller, E. W. Meyers , and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **215**: 403-410.

Frederick, R. D., K. E. Snyder, P. W. Tooley, Y. Berthier-Schaad, G. L. Peterson, M. R. Bonde, N. W. Schaad , and D. A. Knorr. 2000. Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. Phytopathology **90**(9): 951-960.

Fritz, L. K. , and M. T. Kingsley. 2000. Identification of the citrus canker disease pathogen *Xanthomonas axonopodis* pv. citri by LightCycler-based TaqMan and dual FRET probe assays. American Society for Microbiology, 100th General Meeting: P77.

Gabriel, D. W., J. E. Hunter, M. T. Kingsley, J. W. Miller , and G. R. Lazo. 1988. Clonal population structure of Xanthomonas campestris and genetic diversity among citrus canker strains. Mol. Plant-Microbe Interact. **1**: 59-65.

Gabriel, D. W., M. T. Kingsley, J. E. Hunter , and T. Gottwald. 1989. Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. phaseoli* (ex Smith) to species and reclassification of all *X. campestris* pv. citri strains. Int. J. Syst. Bacteriol. **39**: 14-22.

Gottwald, T. R., G. Hughes, J. H. Graham, X. Sun , and T. Riley. 2001. The citrus canker epidemic in Florida: the scientific basis of regulatory eradication policy for an invasive species. Phytopathology **91**(1): 30-34.

Hartung, J. S., J. F. Daniel , and O. P. Pruvost. 1993. Detection of *Xanthomonas campestris* pv. citri by the polymerase chain reaction method. Appl. Environ. Microbiol. **59**(4): 1143-1148.

Hartung, J. S., O. P. Pruvost, I. Villemot , and A. Alvarez. 1996. Rapid and sensitive colorimetric detection of Xanthomonas axonopodis pv. citri by immunocapture and a nested-polymerase chain reaction assay. Phytopathology **86**: 95-101.

Kingsley, M. T. 2000. Technical Requirements Document: Nucleic acid-based detection and identification of bacterial and fungal plant pathogens. Richland, WA, Pacific Northwest National Laboratory**:** 31.

Kingsley, M. T. , and L. K. Fritz. 2000. Identification of the citrus canker pathogen *Xanthomonas axonopodis* pv. *citri* A by fluorescent PCR assays. Phytopathology **60**(6 suppl.): S42.

Lay, M. J. , and C. T. Wittwer. 1997. Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. Clin. Chem. **43**(12): 1-6.

Lazo, G. R., R. Roffey , and D. W. Gabriel. 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment length polymorphisms. Int. J. Syst. Bacteriol. **37**: 214-221.

Niessen, M. L. , and R. F. Vogel. 1997. Specific identification of Fusarium graminearum by PCR with *goa*A targeted primers. System. Appl. Microbiol. **20**(1): 111-113.

O'Donnell, K., H. C. Kistler, B. K. Tacke , and H. H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum,* the fungus causing wheat scab. Proc. Natl. Acad. Sci. USA **97**: 7905-7910.

Sambrook, J., E. F. Fritsch , and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual/Second Edition. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory.

Schaad, N. W., Ed. 1988. Laboratory Guide for the Identification of Plant Pathogenic Bacteria, 2nd. Edition. St. Paul, APS Press.

Schaad, N. W., A. K. Vidaver, G. H. Lacy, K. Rudolph , and J. B. Jones. 2000. Evaluation of proposed amended names of several pseudomonads and xanthomonads and recommendations. Phytopathology **90**(3): 208-213.

Trail, F. 2000. Fusarium graminearum, the head scab fungus: an update on genetics and genomics. IS-MPMI Reporter**:** 1-2.

Verniere, C., J. S. Hartung, O. P. Pruvost, E. L. Civerolo, A. M. Alvarez, P. Maestri , and J. Luisetti. 1998. Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia. European J. Plant Pathol. **104**: 477-487.

Whiteside, J. O., S. M. Garnsey , and L. W. Timmer, Eds. 1988. Compendium of Citrus Diseases. St. Paul, APS Press.

Windels, C. E. 1992. Fusarium. Methods for Research on Soilborne Phytopathogenic Fungi. L. L. Singleton, J. D. Mihail , and C. M. Rush. St. Paul, APS Press**:** 115-128.

Wittwer, C. T., M. G. Herrmann, A. A. Moss , and R. P. Rasmussen. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques **22**(1): 130-138.

Young, J. M., J. F. Bradbury, L. Gardan, R. I. Gvozdyak, D. E. Stead, Y. Takikawa , and A. K. Vidver. 1991. Comment on the reinstatement of *Xanthomonas citri* (ex Hasse 1915) Gabriel et al. 1989 and *X. phaseoli* (ex Smith) Gabriel et al. 1989: indication of the minimal standards for the genus *Xanthomonas*. Int. J. Syst. Bacteriol. **41**: 172-177.