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DEVELOPMENT OF REAL-TIME PCR METHOD FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF FIVE DIFFERENT *FUSARIUM* SPECIES

A Thesis
Submitted to the Graduate Faculty
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Kakolie Goswami

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DEVELOPMENT OF REAL-TIME PCR METHOD FOR DETECTION,

IDENTIFICATION AND QUANTIFICATION OF FIVE DIFFERENT *FUSARIUM* SPECIES

By

Kakolie Goswami

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ABSTRACT

Goswami, Kakolie, M.S., Food Safety Program, College of Graduate and Interdisciplinary Studies, North Dakota State University, November 2010. Development of Real-Time PCR Method for Detection, Identification and Quantification of Five Different *Fusarium* Species. Major Professor: Dr. Charlene Wolf-Hall.`

Fusarium head blight (FHB) is a fungal disease affecting cereal crops worldwide. FHB involves multiple *Fusarium* species that create food safety concerns by producing mycotoxins such as trichothecenes, zearalenone and moniliformin. Quantitative methods allowing rapid risk assessment of mycotoxigenic *Fusarium* species are needed to detect *Fusarium* spp. Real time quantitative PCR (qPCR) is a fast, sensitive and reliable alternative to conventional culture methods. An objective of the study was to develop TaqMan® based qPCR methods to detect, identify and quantify five different *Fusarium* species associated with FHB, namely *F. graminearum, F. culmorum, F. avenceum, F. poae* and *F. sporotrichioides*. The project was initiated with the selection of a protocol for *Fusarium* DNA extraction. Three different commercially available DNA extraction kits, including FastDNA®, Qiagen® Blood & Tissue kit and Qiagen® DNeasy Plant kit were evaluated for speed, DNA yield and quality. The results showed that FastDNA® kit gave the highest DNA yield in least time. TaqMan® Minor Groove Binder (MGB) probes for *F. culmorum*, *F. poae* and *F. sporotrichioides* were developed using qPCR primers from a previous study. For *F. avenaceum* and *F. graminearum*, TaqMan® based methods already available were used with modified conditions for improved detection. The qPCR methods were tested on a wheat system and the result was a qPCR system that rapidly identified and quantified *Fusarium* species associated with FHB.

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TABLE OF CONTENTS

ABSTRACT iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES ix

INTRODUCTION 1

PROBLEMS ADDRESSED BY THE PROJECT 3

HYPOTHESIS 4

OBJECTIVES 5

LITERATURE REVIEW 6

- Fusarium Head Blight 6
- Economic Impact of the 1993 FHB Epidemic 7
- Causal Plant Pathogen: *Fusarium* 8
- Mycotoxins 11
- Intervention Strategies 17
- Non-PCR Detection Strategies for *Fusarium* 18
- PCR Based Detection of *Fusarium* Species 20
- DNA Extraction 23

MATERIALS AND METHODS 25

Objective 1: To Compare Three Commercially Available DNA Purification Kits for DNA Yield and Quality 25

Objective 2: To Develop Individual Real Time Taqman® Probes For Five Species Of *Fusarium* 31

RESULTS AND DISCUSSION 36

Objective 1: To Compare Three Commercially Available DNA Purification Kits For DNA Yield And Quality 36

Objective 2: To Develop Individual Real Time Taqman® Probes For Five Species Of *Fusarium* 41

FUTURE RESEARCH 49

REFERENCES 51

APPENDIX 1: BACKGROUND INFORMATION ABOUT PCR, qPCR AND PRIMER DESIGN 65

APPENDIX 2: RAW DATA FOR DNA EXTRACTION AND PROBES 72

APPENDIX 3: PRELIMINARY EXPERIMENT TO EVALUATE EFFICACY OF TAQMAN® PRIMER-PROBE SETS DEVELOPED IN THE STUDY FOR USE IN DUPLEX qPCR REACTIONS 82

LIST OF TABLES

Table Page

1. Systematics of *Fusarium* species and their associated mycotoxins produced in cereals. 10

2. LD_{50} and TDI values for mycotoxins isolated from FHB infected grains 12

3. Common targets for PCR amplification in *Fusarium* spp. 22

4. Species, strains and culture media used for the experiments 29

5. Protocol used during DNA Extraction 30

6. Genbank accession number of isolates used for designing primers and checking cross reactivity. 33

7. Primer sequences used in the study. 34

8. Results from a preliminary assessment of DNA extraction kits 36

9. Results of the two way ANOVA to compare the DNA extraction results between three kits 39

10. Average ratio of DNA to protein for kits used during DNA extraction 40

11. Ct and Melt Curve values for Nicholaisen primers 42

12. SYBR Green Ct and Melt Curve using primers from Waalwijick et al., 2004 44

13. Ct values for five species by varying template concentration 1 μM primer concentration, and 100 nM probe concentration 46

14. Ct from artificially inoculated wheat DNA 47

15. Excitation and emission wavelength of commonly used QPCR fluorophores and quenchers 70

16. DNA extraction data from *F. avenaceum* 72

17. DNA extraction results for *F. culmorum* 73

18. DNA extraction results for *F. graminearum* 74

19. DNA extraction results for *F. poae* 75

20. DNA extraction results for *F. sporotrichioides* 76

21. DNA extraction yields (used in Figure 2) 77

22. Cross reactivity against species of *Fusarium* 77

23. GenBank accession number and the strain information 78

24. Ct values after varying the primer concentration 81

25. Result of duplex qPCR reactions with *Fusarium avenaceum* (FA) and *Fusarium culmorum* (FC) 83

26. Duplex qPCR data for *F. culmorum* (FC) and *F. poae* (FP) 84

27. Duplex qPCR data for *F. culmorum* and *F. sporotrichioides* 85

LIST OF FIGURES

Figure | Page

1. Flowchart depicting steps for lysis and protein removal during DNA isolation from *Fusarium* species 28

2. Histogram depicting yield obtained after DNA extraction was performed using three commercially available DNA isolation kits. (Error bars represent the standard deviation) 38

3. Amplification curves for *F. culmorum* (Pink), *F. poae* (Yellow) and *F. sporotrichio-ides* (Blue) using primers from Nicholaisen et al.,2009 43

4. Melting curve for *F. culmorum* (Pink), *F. poae* (Yellow) and *F. sporotrichioides* (Blue) generated by plotting the change in relative fluorescence per unit (ΔRFU) unit time (ΔT) against increasing temperature using primers from Nicholaisen et al., 2009 43

5. Amplification curves for *F. avenaceum* (Pink and light blue) and *F. graminearum* (Red and Dark Blue) using primers from Waalwijick et al., 2004 44

6. Melting curve for *F. avenaceum* (Pink and light blue) and *F. graminearum* (Red and Dark Blue) by plotting the change is relative fluorescence per unit (ΔRFU) by the change in temperature (ΔT) using primers from Waalwijick 2004 45

6. Fluorescent molecules used in PCR: 67

7. Duplex qPCR reactions for *F. avenaceum* (FA) and *F. culmorum* (FC) 84

8. Graph for duplex qPCR reactions of *F. culmorum* and *F. poae* 85

9. Duplex qPCR of *F. culmorum* (FC) and *F. sporotrichioides* (FS) 86

INTRODUCTION

Fusarium head blight (FHB) is a fungal disease that debilitates cereal crops and is caused by *Fusarium* species. Multiple *Fusarium* species are isolated from field samples associated with FHB epidemics. *Fusarium graminearum* is frequently recovered from FHB outbreaks and is considered the primary pathogen (Salas et al., 1997). Other species involved in disease vary geographically and the most common FHB pathogens in midwesteren United States are *Fusarium culmorum, Fusarium avenaceum, Fusarium poae* and *Fusarium sporotrichioides* (McMullen et al., 1997, Salas et al., 1997). The difference in prevalence and aggressiveness is due to genetic variation and dissimilar growth requirements of *Fusarium*. FHB causes the wheat florets to appear tan or bleached. In severe infections the entire wheat head or spikelets become blighted, discolored or bleached. The wheat grains infected with FHB have lowered weight and reduced quality. The yield losses accompanying FHB are tremendous as seen from the 1993 epidemic, when wheat yield was reduced from 50 bushels per acre in 1992 to 20 bushels per acre in 1993 (Hollinsworth, 2004).

Mycotoxins like trichothecene, zearalenone, and moniliformin are produced as secondary metabolites by *Fusarium*, developing under favorable conditions. These mycotoxins cause food safety concerns by contaminating feed and food. Ingesting mycotoxins leads to outbreaks of human disease like alimentary toxic aleukia reported in Russia (Wannemacher et al., 1997), or feed refusal in animals (Petska et al., 2005). Pet food containing trichothecene mycotoxins like T2 and deoxynivalenol (DON) have caused acute toxicity and chronic health effects in dogs and cats on Portugal and Poland in 2003 and 2004 respectively (Boerman et al., 2007). The Food and Drug Administration (FDA)

regulates myxotoxin contents by issuing regulations or guidelines. For the trichothecene deoxynivalenol (DON, also known as vomitoxin) the tolerated amount of DON is 1 parts per million (ppm) for finished wheat products destined for human consumption. Feed has a limit of 10 ppm for cattle and poultry and 5 ppm for feed intended for consumption by pigs (Anonymous, 1993).

Conventional methods used to detect *Fusarium* rely on culturing followed by taxonomic identification which is slow and labor intensive, and may often lead to false negatives due to misidentification of species. The methods also suffer from other limitations, including the inability to quantify species specific biomass and have limited sensitivity. Current research methods have shifted to molecular methods. It is possible to simultaneously detect as well as quantify *Fusarium* using quantitative real time polymerase chain reaction (qPCR). DNA used for QPCR should be free of proteins that interfere with reactions. Commercially available DNA extraction kits can give high DNA yield in short duration and have low levels of contaminants that inhibit PCR.

PROBLEMS ADDRESSED BY THE PROJECT

Fusarium head blight (FHB), or scab, has multifold implication as it effects safety, crop yield and quality. The economic impacts of FHB have been tremendous, as experienced during the 1993 tri-state epidemic, which led to $2.7 billion in losses (Nganje et al., 2002). Food safety concerns associated with FHB arise due to mycotoxins produced by the causal organism that accumulate in the grain. Mycotoxins including type A and type B trichothecenes, zearalenone (ZEA) and moniliformin (MON) are a cause of concern. Historically, consuming overwintered FHB infected grains containing trichothecenes have led to outbreaks of alimentary toxic aleukia (ATA) and akakabi byo in humans (Wannemacher et al., 1997). ZEA is an estrogen analogue that causes reproductive disorder in animals (Petska et al., 2005, Rotter et al., 2001, Wannemacher et al., 1997) while MON can affect muscle tissues and block the action of enzymes (Burmeister et al., 1978).

It is essential to have a rapid testing method that can monitor the disease progression, allow risk assessment and help validate intervention strategies for FHB. A number of detection methods for *Fusarium* species are currently available. These methods detect the organism or metabolites (mycotoxins). Only qPCR strategies allow simultaneous identification and quantification of individual *Fusarium* species. QPCR methods exist for some of the mycotoxigenic *Fusarium* species causing FHB, but not for all. Therefore, the goal of this study was to develop and validate QPCR based protocols that can be used for the five main *Fusarium* species causing FHB in Midwestern US.

HYPOTHESIS

Quantitative real time PCR is an efficient and accurate method to identify and quantify mycotoxigenic *Fusarium* species that cause Fusarium head blight.

OBJECTIVES

1. To compare three commercially available DNA purification kits for *Fusarium* DNA yield and quality.
2. To develop individual real time TaqMan® probes for five species of *Fusarium*.

LITERATURE REVIEW

Fusarium Head Blight

Fusarium head blight (FHB), or scab, is a plant disease that affects cereal crops such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereal*), oat (*Avena sativa*), and occurs less frequently in rice (*Oryza sativa*) (Goswami et al., 2004, McMullen et al., 1997. Nelson et al., 1997, Osborne et al., 2007 Marasas et al., 1984). *F. graminearum* and *F. culmorum* have been the most prevalent species causing FHB in North America (Salas et al., 1997, Walter et. al, 2010). On the other hand, *F. poae*, *F. avenaceum* and *F. sporotrichioides* have been implicated in FHB outbreaks in Europe (Salas et al., 1997, Vogelsgang et al., 2007). The disease is characterized by dark purple to black necrotic lesions seen on the outer surface of wheat florets and glumes (McMullen et al., 1997, Osborne et al., 2007) and premature bleaching of wheat heads (Walter et. al 2010). The peduncle of the wheat plant becomes discolored and the inflorescence has a bleached or tanned appearance (Goswami et al., 2004, McMullen et al., 1997).

Fusarium infects by synchronizing the release of its propagules with the time of flowering of wheat. The released *Fusarium* conidia have a sticky cell wall that facilitates adhesion to the wheat host and prevents displacement (Walter et al., 2010). After adhesion, the first symptoms appear in the middle of the head, spreading to other parts of the plant. The fungus can either enter passively from the stomata or actively by direct penetration of the apical floret. The mycotoxins released in the process of colonizing the host can inhibit protein synthesis (Rotter et al., 1995, Wannenmacher et al., 1997). This results in damage to the plasma membrane and ribosomes causing further injury to the

host (Walter et al., 2010). Apart from initial colonization, *Fusarium* persists and multiplies in the debris left behind from the previous harvests (McMullen et al., 1999).

Economic Impact of the 1993 FHB Epidemic

FHB epidemics were regular in the Midwestern region of United States and Canada between 1991 and 1996, the most damaging of which was the 1993 tri-state outbreak. The three states of North Dakota, South Dakota and Minnesota as well as regions in Canada were affected (McMullen et al., 1999, Salas et al., 1997) and this was termed as the "greatest loss due to any plant disease in North America in a single year" (McMullen et. al, 1999). It resulted in a total monetary loss of $ 2.7 billion including direct and indirect losses in all cereals (Nganje et al., 2002), out of which wheat alone accounted for $86 million. The yield loss in wheat was 176 million bushels including 95 million bushels that were lost in North Dakota, resulting in a 45% dip in wheat yield in that year. South Dakota and Minnesota suffered a loss of 18 million bushels and 43 million bushels of wheat respectively. Disease spread was 4 million hectares of planted area. The remaining wheat grains that were harvested from infected fields had low test weight because the grain within the awn had atrophied (McMullen et al., 1999). Seventy percent of the wheat kernels sampled during the epidemic were positive for mycotoxins such as type A and B trichothecenes, zearalenone, and moniliformin. The cumulative mycotoxin content was as high as 44 ppm (McMullen et al., 1997). The FDA guideline, set in 1993 for allowable DON content was 1 ppm in finished wheat products destined for human consumption (Anonymous, 1993),but the average content of DON was 4.7 ppm in

Minnesota, 2.7 ppm in North Dakota, and 3.7 ppm in South Dakota (McMullen et al., 1997)

Causal Plant Pathogen: *Fusarium*

Fusarium species belong to kingdom fungi, phylum Ascomycota, subphylum, Pezizomycotina, class Sordariomycetidae order Hypocreales and family Nectriaceae. The genus *Fusarium* includes a wide range of phytopathogenic as well as mycotoxigenic filamentous fungi. Over 61 species of *Fusarium* are found globally, of which 35 are known to be mycotoxigenic (Hughes et al., 1999). In the tri-state outbreak of 1993, the primary pathogen implicated was *Fusarium graminearum,* which was present in 62 to 64% of infected barley kernels; other species detected included *Fusarium poae* (13-20%), *Fusarium sporotrichioides* (10-17%), and *Fusarium avenaceum* (6-10%) (Salas et al., 1997). All the five species mentioned have a unique mycotoxigenic profile and need to be characterized individually to develop an understanding of the disease (Salas et al., 1997, Waalwijick et al., 2003).

Although *Fusarium* species are considered soilborne pathogens, some members of the genus colonize roots through soil or infect aerial plant parts giving rise to diseases like scab, crown rot and head blight (Nelson et. al 1994). Depending on the species, *Fusarium* may reproduce sexually through ascospores or asexually by producing microconidia, macroconidia, and/or thick walled asexual resting spores called chlamydospores (Nelson et al., 1994, Walter et al., 2010). Most *Fusarium* species have a wide geographical distribution, and occur in both tropical and temperate regions (Nelson et al., 1994; Marasus

et al., 1984). The systematics and mycotoxin profile of *Fusarium* species commonly associated with FHB are summarized in Table 1.

Fusarium graminearum Schwabe. *F. graminearum* is the principal pathogen involved in FHB. The sexual stage of *F. graminearum* is *Gibberella zeae* and Ascospores produced during the sexual stage are the primary inoculum for FHB infections. Asexual spores include slow developing macroconidia and chlamydospores. Microconidia are absent in F. *graminearum* (Nelson et al., 1984). Taxonomically, *F. graminearum* was subdivided into two groups. Group I caused crown rot disease and was heterothallic (different sexes found on different individuals). Group II was homothallic (reserves for sexual reproduction in the same individual) and was the causal organism for FHB. Group I was later assigned the species *F. pseudograminearum*. The two species are morphologically identical and require molecular markers for distinction (Leslie et al., 2007).

Fusarium culmorum Smith. *F culmorum* is also widely distributed across the globe but is more prevalent in the cool temperate regions. Recent trends have indicated an epidemiological shift towards dominance of *F. graminearum* (Ward et al., 2008). In addition to being a major cause of FHB, *F. culmorum* also causes root rot, seedling blight and foot rot (Nelson et al., 1984) Reproduction is strictly asexual through conidia; and the perfect stage is absent. Microconidia are absent, however macroconidia and chlamydospores are present (Nelson et al., 1984).

Fusarium sporotrichioides Sherb. *F. sporotrichioides* can grow at very low temperature of -2°C and at a low water activity of 0.88, which makes it persistent in

Table 1: Systematics of Fusarium species and their associated mycotoxins produced in cereals.

Species	Section	Perfect Stage	Conidia	Mycotoxins	References
F. avenaceum	Roseum	*Gibberella avenacea*	Microconidia, Macroconidia	Moniliformin, Nivalenol, Beauvericin, Enniatins	Bottalico and Perrone, 2002, Marasas et al.,1984, Nelson et al.,1984, Salas et al., 1997, Uhlig et al., 2007, Vogelgsang et al., 2008
F. culmorum	Discolor	-	Macroconidia Chlamydospores	Deoxynivalenol, Zearalenone, Nivalenol, 15-Acetyldeoxynivalenol, zeraralenols (α and β isomers)	Bottalico and Perrone, 2002, Nelson et al., 1984, Salas et al., 1997, Wagacha et al., 2007
F. graminearum	Discolor	*Gibberella zeae*	Macroconidia Ascospores	Deoxynivalenol, Zearalenone, Nivalenol, 15-acetoxydeoxynivalenol, Fusarenone-X	(ottalico and Perrone, 2002, Nelson et al., 1984, Marasas et al., 1984, Salas et al., 1997
F. poae	Sporotrichella	-	Microconidia Macroconidia Chlamydospores	HT-2 toxin, T-2 toxin, and neosolaniol, Enniatins Diacetoxyscirpenol, Monoacetoxyscirpenol Scirpentriol, fusarenone-X	(ottalico and Perrone, 2002, Nelson et al., 1984, Salas et al. 1997, Vogelsgang et al., 2008
F.sporotrichio-ides	Sporotrichella	-	Microconidia Macroconidia, Chlamydospores	T-2 toxin, HT-2 toxin, T-2 tetatol, neosolaniol	(ottalico and Perrone, 2002, Nelson et al., 1984, Salas et al., 1997

overwintered wheat as well as debris from pervious harvests (Moss M. O. 2002). It bears abundant micro and macrocondia as well as chlamydospores. It is often confused with another *Fusarium* species, *F. chlamydosporum*. However, the presence of two types of microconidia, one spindle shaped and one oval or pear shaped makes *F. sporotrichioides* distinguishable from *F. chlamydosporum* (Nelson et. al 1984). *F. sporotrichioides* occurs in temperate to cold areas like northern Europe and USA, Canada, Japan and USSR (Marasas et al., 1984).

Fusarium poae Wollenw. *F. poae* is commonly implicated in FHB infections. There has been an increase in the occurrence of *F. poae* isolated from infected cereals in the recent times (Volsgang et al., 2008). It has abundant microconidia, rare macroconidia and chlamydospores. Surveys of FHB infected wheat done in 2003, in Finland, Germany, Hungary, Ireland, Italy, and United Kingdom identified *F. poae* to be the most consistently occurring species among other *Fusarium* species detected (Vogelsgang et al., 2008).

Mycotoxins

Fusarium species produce a wide range of toxic secondary metabolites that contaminate food and feeds of plant origin (Moss M.O., 2002). Mycotoxins do not influence *Fusarium* growth and metabolism directly; however they may give competitive advantage and allow better pathogen survival in the host (Desjardin et al., 1993; Neissen L., 2005; Richard, 2007; Wannemacher et.al. 1997). The major classes of mycotoxins produced during FHB outbreaks include trichothecenes, zearalenone, and moniliformin (McMullen et al., 1997, Salas et. al, 1997). The mycotoxins contaminate food intended for human consumption as well as animal feed. Mycotoxins like trichothecenes survive

temperatures of up to 100°C, and inactivation requires temperatures of 500°F for 30 min or 900°F for 10 min (Wannemacher et al., 1997).

The amount of mycotoxin produced and accumulated in a grain sample is influenced by several factors including environmental conditions, degree of contamination and aggressiveness of the isolates. The environmental factors influencing production include moisture, oxygen and type of substrate (Meerdink G. L., 2002). The toxicity of mycotoxins is evaluated using lethal dose 50 (LD_{50}), which is the dose at which half of the test population perishes. The LD_{50} and the tolerable daily intake (TDI) of mycotoxins are summarized in Table 2.

Table 2: LD_{50} and TDI values for mycotoxins isolated from FHB infected grains

Mycotoxin	LD_{50} (mg/kg bodyweight)	TDI (µg /kg bodyweight)	Test Organism (Route of exposure)	Reference
T-2 toxin	5.2	0.06	Mouse (Intravenous)	Lawley et al., 2008, Wannemacher et al., 1997
Deoxynivalenol	46	3.0	Mouse (Oral)	Petska et al., 2005, Rotter et. al 1995
Nivalenol	38.9	0.7	Mouse (Intra-peritoneal)	Anonymous, 2000
Moniliformin	5.4	-	Chicken (Crop-intubation)	Burmeister et al., 1978
Zearalenone	10000	0.1	Rat (oral)	Moss. M. O. 2002

Trichothecenes. They are a family of chemically related mycotoxins that contain a sesquiterpene epoxide group. The chemical structure comprises of a tetracyclic 12,13-epoxytrichothecene skeleton, with different functional groups, dividing the trichothecenes into four types. Trichothecene A, B, C and D are classified based on the functional group attached to the basic chemical structure (Pronk et al., 2002, Wannenmacher et al., 1997). Type A includes highly toxic forms like T-2, neosolanilol, diacetoxyscirpenol and HT 2 toxins, type B trichothecenes includes lesser toxic DON, 3-acetyl DON, 15-acetyl DON, nivalenol (NIV), and 4-acetyl NIV. Type C encompasses toxins containing a second epoxide ring, while type D includes atratoxins G and H that have a macrocyclic ring. The primary mechanism of trichothecene toxicity is by protein synthesis inhibition and immune system suppression (Lawley et al., 2002, Wannemacher et al., 1997).

T-2 Toxin. T-2 is a Type A trichothecene which is produced by *F. sporotrichioides*, *F acuminatum* and *F. poae* (Marasas et al., 1984), as well as *F. equiseti* (Nelson et al., 1984). The T-2 toxin damages bone marrow and reduces white blood cell counts causing a condition known as aleukia. Alimentary toxic aleukia (ATA) is a human disease caused by ingesting FHB infested grains (Moss M. O. 2002, Wannenmacher et al., 1997). The symptoms shown in the early stages of ATA include nausea, vomiting, headaches and necrotic lesions in the mouth, throat, gastric and inner mucosa. In the later stages, localized rashes appear on the skin and progressively become numerous and dispersed. After prolonged ingestion of the mycotoxins, the patient attains severely retarded immunity and becomes susceptible to secondary infections like pneumonia. Ulcers and gangrene develop in the larynx and can also cause death by strangulation

(Marasus et al., 1984, Moss, 2002, Nelson et al., 1997, Wannemacher et al., 1997). An ATA outbreak was documented during World War II in Russia (Wannemacher et al., 1997). It has been shown that mycotoxins T-2 and HT-2 showed synergism with DON (Kimura et al., 2007, Larsen et.al. 2004, Nelson et al., 1994, Petska et al., 2005). T-2 and HT-2 also tend to show additive effects with other *Fusarium* toxins such as diacetoxyscirpenol. T-2 is often studied together with HT-2 as it rapidly metabolizes to HT-2 in the gut (Lawley et al., 2002). The threat of T-2 toxin contamination reemerged in 2002, when it was found that 11% of European cereal grain samples contained T-2 toxin at low concentrations (Moss, 2002). T-2 and HT-2 were also detected by Schollenberger et al, 2005, while testing various cereal based foods.

Deoxynivalenol. DON are a type B trichothecene produced by *F. culmorum, F. graminearum, F. acuminatum, F. crookwellense, F. roseum* (Marasas et al., 1984, Vesonder et. al 1982) and some strains of *Microdochiium nivale* (Logrieco et. al 1991, Teich A. H, 1989). DON is also called vomitoxin due to emesis that follows ingestion. Like all trichothecenes, the mechanism of toxicity is by inhibiting protein synthesis and immune function (Canady et al., 2001). DON binds to the 60S subunit of cellular ribosome and inhibits protein synthesis by interfering with peptidyltransferase activity (Rotter et al., 1995 Canady et al., 2001, Kimura et al., 2007, Smith et al., 1997). Immunosupression is observed in mice at a concentration of 0.25 mg/kg body weight (Canady et al., 2001). Recently, the role of DON in inhibiting cell signaling was also studied and DON was found to alter the brain chemicals that mediate feeding behavior, causing anorexia and feed refusal in animals (Amuzie et al., 2009).

DON is not reported to show any *in vivo* carcinogenic, teratogenic or mutagenic properties, except some cases of chromosomal aberration seen *in-vitro* (Canady et al., 2001). It is less toxic than other potent trichothecenes, such as T-2, HT-2 and fusareon X (Rotter et al., 1995). However, DON is economically more important due to its prevalence and heat stability. DON persists in grains during storage and is unaffected by high temperature and pressure during processing (Wolf-Hall et al., 1999). In Canada, the acceptable limit for DON in grains is 2 ppm in unclean soft wheat (Canady et al., 2001). In United States it is 1 ppm for bran, flour and germ destined for human consumption, and for poultry feed and domestic animals, the acceptable limit for ingestion are 10 ppm, whereas for swine populations, the level is 5 ppm (Anonymous, 1993). Dogs and cats show similar susceptibility to DON as seen in studies done by Hughes et al., 1997. Feed refusal was observed when 4.5 ± 1.7 mg of DON contaminated feed was fed to dogs and the same effects were observed in cats when 7.7 ± 1.1 mg of DON was added in feed (Hughes et al., 1999).

Nivalenol. NIV is a type B trichothecene, primarily produced by *Fuarium nivale* as well as *F. poae, F. culmorum*, and *F. gramineraum*. Apart from obstructing protein synthesis (Wannemacher et al., 1997, Moss M. O. 2002), NIV also acts as a potent nucleic acid synthesis inhibitor as seen in studies on Chinese hamster V79 cells (Anonnymous, 2000). At doses of 50-100 µg, DNA damage was seen in the form of strand breaks, sister chromatids and chromosomal aberrations. Hematoxicity appeared in the form of erythropenia and mild leukopenia was observed at doses of 3.5mg/kg body weight in mice (Pronk et al. 2002, Li et al., 2005)

Moniliformin. MON is known to be produced by some strains of *F. avenaceum* (Uhlig et al., 2007). High concentrations of MON are also produced by *F. moniforme, F. proliferatum, F. subglutinans*, and *F. thapsinum*, whereas low concentrations are produced by *F. verticillioides, F. acuuminatum,* and *F. equiseti* (Nelson et al., 1984, Marasas et al., 1984). Apart from wheat, MON is also found in oats, millet, sorghum and barley (Burmeister et al., 1978, Desjardins et al., 2002). Chemically, MON is a small ionic molecule that occurs as sodium or potassium salt, and is difficult to isolate due to high water solubility. MON causes pathological effects on cardiac tissues (Desjardins et al., 2002) and MON inhibits gluconeogenesis by restricting the action of enzymes like aldose reductase and reductase (Uhlig et al., 2007). Severe leg deformities are developed in poultry when the population is exposed to MON (Burmeister et al., 1978). MON causes muscular weakness and acidosis in poultry by targeting cardiac and skeletal muscles. It has been associated with some human outbreaks of Keshan disease in China (Uhlig et al., 2007). The symptoms associated with ingesting MON include depression, ataxia and weakness with terminally labored respiration (Burmeister et al., 1978).

Zearalenone. ZEA is known to co-exist with DON because it is produced under similar conditions by the same causal organisms i.e. *F. graminearum* and *F. culmorum* (Richard, 2007). ZEA occurs with its co-metabolites α-zearalenol, β-zearalenol, and 4-acetylzearalenone. Other species producing ZEA include *F. equiseti, F crookwellense,* and *F. semiticum*. Zearalenone has a high LD50 (Table 2), and is not associated with mortalities; however, being an estrogen analoge, ZEA causes hormonal imbalances by mimicking 17 β esterdiol, which is the hormone produced in the ovaries (Bhunia A. K., 2009). ZEA binds to the estrogen receptors in the ovary causing reduced fertility in

females (Desjardins et.al. 2003). The symptoms include enlargement of the mammary glands in both male and female pigs. ZEA affects the organs in the genital tract of female pigs causing vulvovaginitis (Moss M.O. 2002). The ovaries degenerate and there is reduction in the litter size. In males degeneration of the testis as well as infertility occurs (Desjardins A. E., 2003).

Intervention Strategies

Control of *Fusarium* spp. and their mycotoxins can be done at the pre-harvest and post-harvest stages. Pre-harvest control involves use of resistance to initial infection and reduction of disease spread in the field.

Pre-Harvest Control. Morphological properties of wheat that impede pathogen colonization include extent of flower opening and plant height. Biochemically, the plant escapes pathogen invasion by secreting waxes and having spikelet tissues. The physiological properties such as flowering time and duration, or the absence of awn acts as an added defense against fungal infection in developing wheat (Walter et al., 2010). Grain variety may also control susceptibility to infection as shown in studies, where soft kernel wheat was found to be more vulnerable to *Fusarium* infections (Magan and Aldred, 2007). In addition, the time of sowing and harvest influences *Fusarium* growth as demonstrated by increased ZEA concentrations in late maturing hybrid wheat varieties. Growing resistant cultivars, appropriate use of fungicide, agronomic practices such as deep ploughing to remove residual fungi and crop rotation can control existing fungi and prevent carryover from previous harvest and plant debris (Magan and Aldred, 2007).

Fungicides are also commonly applied to control the spread of fungi; however the use of fungicides may induce production of mycotoxins (Aldred and Magan, 2004)

Post-Harvest Control. The most effective way of managing *Fusarium* in stored grain is by controlling some critical points during harvesting, drying and storage. The hazard analysis critical control points (HACCP) system for controlling the production of DON has been reviewed by Aldred et al., 2004. The Critical Control Points include controlling moisture content of the grains and maintaining them at <14.5%. Additionally, modifying the atmosphere by lowering the oxygen concentration to >0.14% and increasing the carbon dioxide content to >50% also controls the growth of *Fusarium* and mycotoxin production. Fumigation with ammonia and sulfur dioxide controls *Fusarium* during storage. Treatment of wheat grains with ozonated water can serve as a means to remove mold (Dhillon et al., 2009). Most of the grossly contaminated grains are screened out and removed during the milling process (Aldred and Magan, 2004).

Non-PCR Detection Strategies for *Fusarium*

Strategies for detecting the presence of *Fusarium* include culturing techniques, biochemical analysis of mycotoxins, immunological detection using enzyme linked immunosorbent assay (ELISA) or nucleic acid based methods. Each method possesses some limitations and research focus was on developing rapid techniques and overcoming identification related drawbacks (Neissen et. al, 2007).

Culturing Techniques. These are primarily based on morphological characteristics and are accomplished with live propagules. Identification of *Fusarium* species based on this method is time consuming and requires significant amount of skill, experience and

expertise. Distinction of *Fusarium* at the species level is often difficult as closely related species may show strong morphological similarities and have conflicting taxonomic categorization (Edwards et al., 2002). Moreover, there are differences in growth rate, so some of the species present in mixed cultures may be outcompeted, resulting in poor representation and false negatives. Certain *Fusarium* species express their identifying characteristics in select media where stringent growth conditions are required for their manifestation. In the absence of such conditions, cultures mutate and characters required to distinguish *Fusarium* are not expressed (Filion et al., 2003). This causes confusion that can lead to misidentification and incorrect assessment of toxigenic potential (Niessen et al., 2007, Nelson et.al. 2002).

Immunological Methods. These methods include the use of ELISA which uses specific antibodies to bind to either the organism or the mycotoxin antigen. The binding results in a color reaction indicating a positive mycotoxin presence. ELISA has introduced promptness in sample screening, allowing parallel processing of multiple samples. This method is used as a quick screen for *Fusarium* mycotoxins and several commercial kits are available. No organic derivatives are produced, eliminating the cleanup step. This method is also quantitative. Species specific assays are not commercially available yet, however this has been a research focus for some time. Hill et al., 2006, developed a method for detecting and quantifying *Fusarium* in barley; however this method suffers from the limitation of having high prevalence of false positives and not being species specific.

PCR Based Detection of *Fusarium* Species

Background information about PCR and primer designing is furnished in Appendix 1. Reviews of advancements in PCR techniques for FHB causing *Fusarium* species has been published by Nicholson et al., 2003 and Mule et al., 2005. Multiple regions in the *Fusarium* genome have been exploited as targets for PCR reactions. Mycotoxigenic *Fusarium* species can be identified and distinguished from others by targeting the *tri* gene cluster that regulates trichothecene mycotoxin production (Desjardins et al., 1993, Desjardins et. al 2003). The *tri* gene cluster has been reviewed by Kimura et al. 2007, and this region is a common target for identifying trichothecene producers. Bluhm et al., 2002 designed a multiplex TaqMan® assay using the *tri*6 gene from this cluster. The method was able to distinguish trichothecene producing *Fusarium* species from fumonisin producers. The *Tri* gene cluster shows positive correlation between amount of mycotoxin produced and *Fusarium* DNA quantified (Waalwijick et al., 2004); however, the mycotoxin gene targets rarely give information about the species. A review of the commonly used PCR techniques for detection of genes associated with mycotoxin production was done by Edwards et al., (2006) and Niessen, (2007).

Another target for distinguishing *Fusarium* at the genus level includes regions of ribosomal DNA (rDNA). The rDNA consist of conserved as well as variable regions exploited for distinguishing related taxonomic groups (Edwards et al., 2006). There are two internal transcribed spacer (ITS) regions, ITS1 and ITS2 which flank a gene coding for 5.8S ribosomal ribonucleic acid (RNA) (Edwards et al., 2006). Traditional PCR primers that can identify and distinguish *Fusarium* from other species have been developed by targeting the ITS region. However, there is limited polymorphism in the ITS

region, and it cannot be used to distinguish *Fusarium* at the species level (Nicholaisen et al., 2009). Thus, the ITS is a good marker for distinguishing *Fusarium* species from other fungi and mycotoxigenic species, however, the region cannot intra-specifically discriminate *Fusarium*. The inter genomic spacer region (IGS) is another 2000 bp region commonly targeted for PCR assays. The IGS region separates repeated ribosomal units (Llorens et al., 2006), and is present between 28S and 18S subunits. This region is considered suitable for distinguishing *Fusarium* at the species level, and Burlakoti et al., 2007 developed a detection method based on the intergenic spacer (IGS) region. Recent studies have focused on establishing a phylogenetic relationship between species based on the IGS region (Llorens et al., 2006). Primers that can distinguish between *Fusarium* species were designed for *F. graminearum, F. culmorum, F. equiseti, F, sporotrichioides,* and *F. poae*. There are high levels of sequence variability found at the IGS region among the different *Fusarium* species (Jurado et al., 2005); however the availability of sequences for this region in public databases is limited.

The most commonly used region for identification of *Fusarium* species is the translation elongation factor 1 α (EF1α). This region has been widely studied and no orthologous sequences have been detected so far, which has made EF1α the preferred target region for distinguishing *Fusarium* at the species level using PCR. Moreover, a comprehensive database of sequences from the Fusarium EF1α is available at http://fusarium.cbio.psu.edu. A review of the advantages of using EF1α was done by D. M. Geiser, 2004. A summary of commonly used targets for species specific PCR assays for *Fusarium* species is presented in Table 3.

Table 3: Common targets for PCR amplification in *Fusarium* spp.

Species	Target region	PCR	Reference
F. avenaceum	Sequence characterizing RAPD fragments (SCAR)	End-point	Schilling et al., 1999
	ITS	qPCR	Mishra et al., 2003
	Genomic DNA	qPCR	Waalwijick et al., 2004
	EF1α	qPCR	Nicolaisen et al., 2008
F. culmorum	RAPD	End-point	Nicolaisen et al., 1998
	SCAR	End-point	Schilling et al., 1999
	ITS	qPCR	Mishra et al., 2003
	Genomic DNA	qPCR	Waalwijick et al., 2004
	IGS	End-point	Jurado et al., 2005
	IGS	End-point	Jurado et al., 2005
F. graminearum	RAPD	End Point	Parry and Nicholson 1996
	Genomic DNA	qPCR	Waalwijick et al., 2004
	β tubulin region	TaqMan®qPCR	Reicher et al., 2004
	IGS	End-point	Jurado et al., 2005
	tri5-tri6 and IGS	End-point	Li et; al. 2008
F. poae	Genomic DNA	qPCR	Waalwijick et al., 2004
	tri 5	End-point	Bluhm et al., 2004
	EF1α	qPCR	Nicolaisen et.al 2008
	IGS	End-point	Jurado et al., 2005
F. sporotrichioides	*tri* 5	End-point	Neissen et al., 2004
	ITS	qPCR	Mishra et al., 2003
	IGS	End-point	Jurado et al., 2005
	tri 13	End-point multiplex	Demeke et al., 2005

DNA Extraction

Isolation of DNA is a critical step for successful PCR amplification (Fredlund et al., 2008). PCR relies on DNA template purity and quality, which is determined by the effective retrieval of DNA from the cell and absence of PCR inhibitors. The DNA isolation method should have the ability to lyse the fungal cell and recover ample quantities of DNA (Fredricks et al., 2005).

The fungal cell wall is remarkably different from plant or animal cells, and the atypical characteristic of fungi places them in the "fifth kingdom" according to Whittaker's classification. The components in the cell wall such as chitin microfibrils, glucans and other polymers hamper recovery of nucleic acids (Adams, 2004; Freidricks et.al. 2005). This often necessitates the use of additional mechanical lysis step to ensure that the cell wall ruptures properly, and DNA is released into the extraction buffer (de Nijs et al., 1996). Proteinase K is added to the lysate to remove proteins that act as PCR inhibitors. After lysis and removal of membrane lipids and proteins, additional steps include alcohol based precipitation of DNA, washing and elution (Fredlund et al., 2008, Fredricks et al., 2005).

Modifications of the lysis protocols and use of varied methods have been done to enhance the efficiency of fungal DNA extraction (Cenis, 1992, Dean et al., 2004; de Nijs., 1996; Fredlund et al., 2007). Some modifications used by researchers include use of sonication (Fredlund et al., 2004), freeze drying, (Filion et al., 2003), bead milling (Dean et al., 2004) or enzymatic lysis using lysis buffers in commercial kits. Fredricks et al., 2005 compared six DNA extraction methods for *Candida albicans* and *Aspergillus fumigatus* and the results indicated that lysis performed by mechanical agitation gave

higher yields. Haugland et al., 1999 performed another study that compared five different lysis methods on *Aspergillus versicolor, Penicillium chrysogenum, Stachybotrys chartarum, Cladosporium herbarum* and *Alternaria alternate*. The results indicated that the glass bead milling method gave the maximum yield (Haugland et al., 1999). Fredulund et al., 2008 conducted a study to check the DNA extraction efficiency of different commercially available kits using *F. graminearum* and *F. culmorum*, and the results indicated that commercially available Qiagen® Dneasy Plant kit gave highest yields.

MATERIALS AND METHODS

Objective 1: To Compare Three Commercially Available DNA Purification Kits for DNA Yield and Quality

Experimental Design and Statistics. The randomized complete block design (RCBD) was used for the DNA extraction experiment. In this design, similar units were grouped together into blocks that included the five species *F. avenaceum, F. culmorum, F. graminearum, F. poae* and *F. sporotrichioides*. The variable was the commercially available DNA extraction kits; FastDNA® Kit, Qiagen® DNeasy Blood & Tissue Kit and Qiagen® DNeasy Plant Kit (Mendelhall et al., 2003). The number of reactions were species (duplicate/species) × kits = 5 × 2 × 3 = 30 reactions. The experiments were repeated 3 times, hence a total of 90 (30 x 3 extractions were conducted.

Validation experiments were done by inoculating autoclaved wheat with a conidial suspension from each species. DNA was isolated from 5 samples of each species and one sample of autoclaved wheat, which served as the control. Thus the total number of reactions for the validation experiments were; 30 (5 x 6) extractions. The yield was reported based on the absorbance in a Nanodrop™ spectrophotometer (ND 1000, Thermo Scientific Inc. Wilmingtom DE, USA). Nucleic acid absorbs light of 260 nM, and proteins at 280 nM. 1 µL of sample was loaded to the Nanodrop™ spectrophotometer, and absorbance at 260 nM gave the quantity of DNA present in the representative sample. The 260/280 ratio was used to evaluate the DNA quality and results were reported as nanograms per microliter (ng/µL). Statistical analysis was done using Microsoft Office

Excel 2010 and GraphPad software. The statistical significance for evaluating the difference in DNA yield for each species was done using a two way ANOVA.

DNA Extraction from Pure Cultures. The cultures of *F. avenaceum* (R-04608), *F. culmorum* (FRC # R-6566), *F. graminearum* (R-6574), *F. poae* (T-0487) and *F. sporotrichioides* (T-0348) were obtained from the *Fusarium* Research Center (FRC), Pennsylvania State University, Park (PA, USA) and other species and strains used for testing the cross reactivity of PCR primers were either obtained from the FRC or the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, IL. The strain number and media information appear in Table 4.

Fusarium species were first grown on homemade half strength potato dextrose agar (HPDA) (Salas et al., 1997) for 7 days. Conidia were harvested by flooding plates with 3 ml of sterile double distilled (dd) water and filtered through three layered sterile cheese cloth to make a suspension. The conidial suspension was transferred to sterile screw-capped test tubes with 15 ml potato dextrose broth (PDB) (EMD chemicals, Gibbstown NJ, USA). Incubation was at 25°C, with 12 hour photoperiods for 5-7 days. The mycelia were harvested, and sterile Whatman's filter paper #1 was used to remove excess moisture by blotting.

100 mg of harvested mycelia destined for DNA extraction were flash frozen in liquid nitrogen and ground using a tissue grinder. Lysis buffers from individual kits were added to the Lysing Matrix A (Qbiogene, Irvine CA, USA) and the sample was homogenized using the FastPrep 24 Homogenizer (Qbiogene, Irvine CA, USA). The initial steps for lysis, protein removal, and RNA removal are presented in Figure 1, and the DNA extraction was conducted as per the procedure described in Table 5. The three

different commercially available DNA isolation kits used were FastDNA® (Qbiogene, Irvine CA, USA), Qiagen® DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) and Qiagen® DNeasy Plant Kit (Qiagen, Valencia CA, USA). Apart from this a cetyltrimethylammonium bromide (CTAB) based DNA extraction method (Bluhm et. al., 2004) and Wizard® Genomic DNA extraction kit (Promega, Madison, WI, USA) were also tested in preliminary trials, but eliminated from the final experimental design since the other kits gave better DNA yields.

Different DNA isolation kits were compared based on yields recorded. Agarose gel electrophoresis was used to assess DNA quality, by visually estimating DNA smear intensity. 10X TBE (tris-borate buffer) (BioRad, Hercules CA, USA) was diluted to to 1X TBE using dd water. Powdered agarose was added to make a 1% agarose gel. The mixture was heated in a microwave and the electrophoresis was done by running the samples at 100V for 1 hour.

Fusarium Inoculation and DNA Isolation from Autoclaved Wheat. The conidial suspension was prepared by flooding HPDA plates with 5 ml of autoclaved double distilled (dd) water. The plates were gently scraped using a sterile inoculating loop to dislodge and subsequently harvest conidia. The suspension was filtered through a 3-layered sterile cheese cloth to remove mycelia and 1.5 ml of this suspension was transferred to a 2 ml Eppendorf tube. The tubes were centrifuged for 20 min at 16,000 x g and the supernatant in each conidial was discarded. The pellet was re-suspended in sterile dd number water and the conidia suspension was counted using a hemocytometer.

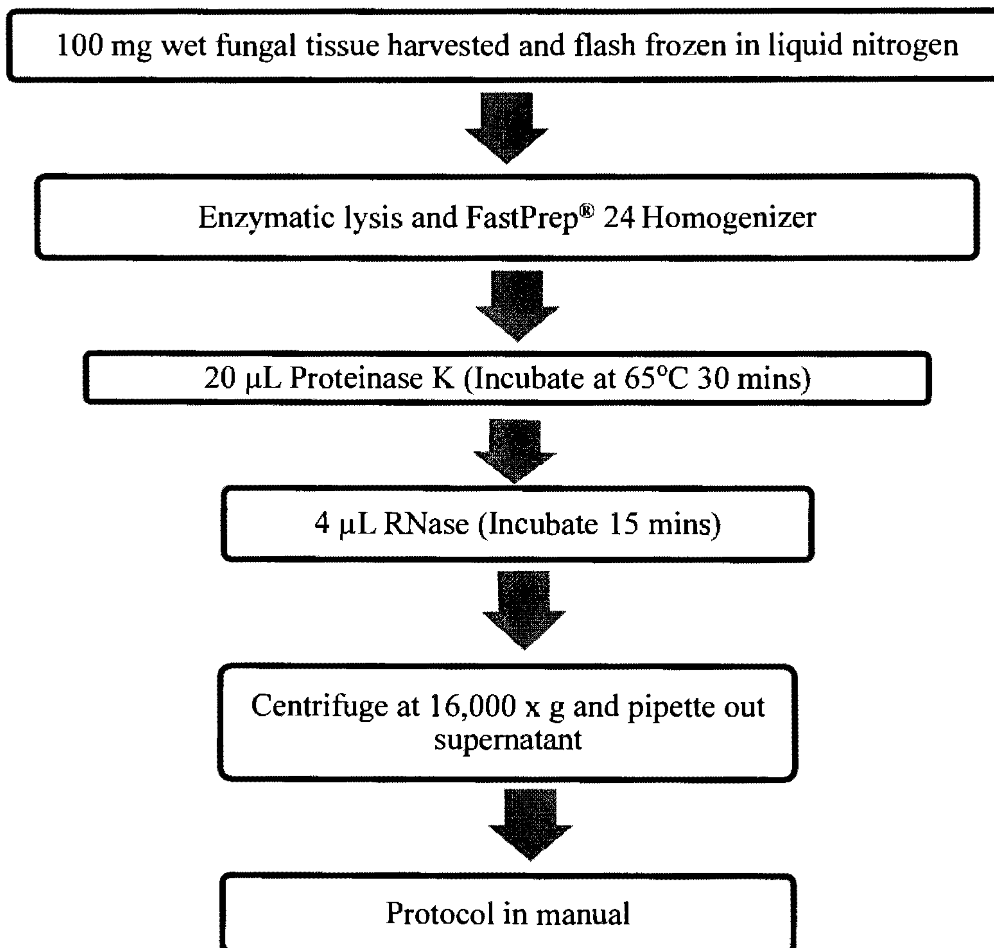

Figure 1: Flowchart depicting steps for lysis and protein removal during DNA isolation from *Fusarium* species

Necessary dilutions were made to obtain a final cell concentration of $5x10^5$ conidia/ml. For the validation experiment, durum wheat kernels were obtained from Casselton Agricultural Station in June 2003 and stored frozen. An amount of 50 g of wheat were soaked in 100 ml distilled water at 1:2 (w/v), at room temperature for 6 h. The excess water was drained out and the wheat was autoclaved at 121°C for 1 h 5 min. The kernels were allowed to cool down to room temperature for about 4 hours and 1 ml of

conidia suspension was added to it (Hoogschagen et al., 2001, Suhanthie et al., 2009). The mixture was placed in sterile 1 L flasks for 7-10 days and sealed with a sterile cotton plug. The flasks were manually agitated at intervals of 2 days to prevent clumping of mycelia.

Table 4: Species, strains and culture media used for the experiments

Species	Strain Number	Culture Medium
F. avenaceum	FRC[a] R-04608	Half strength Potato Dextrose Agar (HPDA)
F. culmorum	R-6566	HPDA
F. graminearum	FRC # R-9821, R-9822, R-9823, R-9824, R-9825, R-9826, R-9827, R-9828, R-6574, R-9830, R-9831, R-9832, PH1(15ADON strain). NRRL[b] # 13383, 26916, 28336, 3184	HPDA
F. poae	FRC#T-0487	HPDA
F. sporotrichioides	FRC # T-0216	HPDA
Geotrichium candidum	Commercial strain	Potato Dextrose Agar (PDA)
Aspergillus flavus	NRRL # 458	Malt Extract agar (MEA)
A. Niger	NRRL-3	MEA
A. ochraceus	NRRL-5175	MEA
Penicillium chrysogenum	NRRL-807	MEA
P. verrucosum	NRRL-965	MEA

[a]FRC = Fusarium Research Center, Pennsylvania State University, University Park, PA.
[b]NRRL: Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, IL.

Table 5: Protocol used during DNA Extraction	
Kit	Protocol
FastDNA®	Lysis Buffer: Cell Lysis buffer: Yeast (CLY) 1000 µL of CLY was added and steps from Figure 1 were followed. Equal quantities of binding matrix was added and incubated for 15 min with gentle agitation. Centrifugation was done at 16,000xg for 15 min and supernatant was discarded. 500 µL of SEWS-M was added and the pellet was re-suspended with the force of the liquid. Centrifugation was done at 16,000 x g for 4 min and supernatant discarded and repeated this step. 150µL of DES™ elution buffer was added to incubat for 15 min at 55°C .Centrifugation at 16,000 x g for 2 min and was done to collect the supernatant, which contained DNA.
Qiagen® DNeasy Blood and Tissue Kit	Lysis Buffer: ATL 800 µL of ATL was added and protocol from Figure 1 was followed. 200 µL of buffer ALwas added after that and the mixture was vortexed and incubated at 70°C for 15 min. 200 µL ethanol was added and mixed by pulse vortex. The mixture was added to the QiaAmp® spin columns and centrifuged at 12,000 x g for 1 min. Added 500 µL AW1 buffer to the column closed the cap and centrifuged 12,000 x g for 1 min.. 500 µL AW2 buffer was added and centrifuged 16,000xg for 3 min. The tube was centrifuged again at 16,000xg after discarding the flow-through and the collection tube. The column twas transferred o a labeled 2 ml centrifuge tube and 105 µL of AE buffer was added. After being incubated for 20 min, and centrifuged for 1 minute at 12000 x g the filtrate contained DNA. Was harvested.
Qiagen® DNeasy Plant Kit	Lysis Buffer: AP1 800 µL of ATL was added and steps from Figure 1 were followed .Added 130 µL of buffer AP2 and incubated on ice. Centrifuged the mixture for 5 min at 16,000 x g. Pipette lysate to the QiaShredder® column and centrifuged for 2 min at 16,000 x g. Transferred the flow thru to a new tube and added 1.5 times the volume of AP3 buffer in. Pipetted half (almost 650 µL) of the mixture to the DNaesy spin column and centrifuged for 1 min at > 12,000 x g. This step was repeated with the rest of the flow-through. The spin column was placed in a new tube and 150µL of buffer AW was added. The mixture was centrifuged for 1 minute at 12,000 x g and the filtrate contained DNA.

Fusarium Infection Rate (FIR). Ten days after inoculations 100 ± 10 kernels were picked and rinsed in 20 ml of dd water. The seeds were soaked in 20% commercial bleach for 30 seconds and rinsed again with dd water. Subsequently, these grains were plated on HPDA plates with 5 grains per plate. FIR is determined by visually assessing the infection after 5 days and using the formula:

$$\frac{\text{Number of infected grains}}{\text{Number of grains plated}} \times 100 = \text{FIR}$$

Fungal DNA Isolation from Autoclaved Wheat. The wheat showing 100% infection was homogenized by grinding in a coffee grinder and was flash frozen in liquid nitrogen. A sanitized pestle and mortar was used to grind the kernels. 200 mg of ground kernels were transferred to lysing matrix A (Qbiogene, Irvine, CA, USA) and 40 µl Proteinase K was added. The initial steps are mentioned in Figure 1 and the procedure is mentioned in Table 5 for FastDNA®. The yield was recorded using a Nanodrop™ spectrophotometer and wheat DNA was diluted 1:10 for the PCR reactions.

Objective 2: To Develop Individual Real Time Taqman® Probes For Five Species of *Fusarium*

Experimental Design. The real time PCR experiment was set up as factorial design. Individual primers were first tested using SYBR Green. TaqMan® probes were designed based on the running conditions of the SYBR Green primers and PCR was performed with the primers and TaqMan® probes. For SYBR Green, five species were tested in duplicates, and reactions were repeated three times i.e. 5 x 2 × 3 = 30 reactions. Cross reactivity of individual primers was tested against different strains mentioned in Table 4 to determine

whether there is any nonspecific amplification of DNA from non target species by the primers; therefore a total of 135 (27 x 5) reactions.

The primer and probe sequences used in the study are mentioned in Table 7. The concentration of primer, TaqMan® probes and DNA template was varied. The primer concentration tested was 1000 nM, 750 nM, 500 nM, 250 nM, and 125 nM, while probe concentrations tested were 100 nM, 75 nM, 50 nM, and 25 nM. The DNA template concentration was varied between 50 ng, 5 ng, 0.5 ng, 0.05 ng, and 0.005 ng. For five species; 5 primer concentration x 4 probe concentration x 5 template concentrations x duplicates = 200 reactions. Cross reactivity was tested for the primer combinations against 27 other strains in duplicate i.e. 270 reactions (5 x 27 x 2).

Validation experiments were performed for every species. Five DNA samples from inoculated and five from the autoclaved wheat (control) were used to validate the PCR. The total number of reactions done in duplicates were; 5 x 6 x 2 = 60 reactions.

Primer Design. The primers for *F. culmorum, F. poae,* and *F. sporotrichioides*, targeting EF1α were selected from a study done by Nicholaisen et al., 2009. The primers and TaqMan® MGB probes for *F. avenaceum* and *F. graminearum* were selected from a study done by Waalwijick et al., 2004. The TaqMan® MGB probes for *F. culmorum, F. poae*, and *F. sporotrichioides* were designed by aligning forward and reverse SYBR Green primers developed by Nicholaisen et. al. 2004, against EF1α sequences of species retrieved from NCBI using nucleotide BLAST (BLASTN) available at http://blast.ncbi.nlm.nih.gov/. The accession numbers of the isolates is summarized in Table 6. The information about the strains selected is given in Table 23 (Appendix 2).

The alignment was done using BioEdit Alignment editor software (Hall, 1999) and Clustal W. TaqMan® MGB probes were designed using Primer Express 4 (Applied Biosystems, Foster City CA, USA). The TaqMan® MGB probes for *F. avenaceum*, *F. poae*, and *F. sporotrichioides* were labeled at the 5' end with VIC® while probes for *F. culmorum* and *F. graminearum* were labeled at the 5' end with 6-FAM. The primers were obtained from Integrated DNA Technologies (Coralville. IA, USA), and the MGB labeled TaqMan® probes were procured from Applied Biosystems (Foster City, CA, USA). The primer sequences are summarized in Table 7.

Table 6: Genbank accession number of isolates used for designing primers and checking cross reactivity.

Organism	GenBank Accession Number
F. avenaceum	EU220414, EF512021, EF512020, EF512019, EF512018, EF105293
F. culmorum	EU220412, EU220410, FJ939672, FJ939671, FJ939670, FJ939669, FJ939668, FJ939667 , FJ939666, FJ939665, FJ939664, FJ939663, EU220410
F. graminearum	EF428714, EF428702 ,EF428701 ,EF428700 , EF428699, EF428698 , EF428697, EF428696, EF428695, EF521161, EF521160, EF521159
F. poae	EU744836, EU744835, EU744834, EU744833, EU744832, EU744831, EU744830, EU744829, EU744828, EU744827, EU744826, EU744825, EU744824, EU744822, EU744821
F. sporotrichioides	AY337442, AY337442, EF521146, AY337442, EF521145

Table 7: Primer sequences used in the study.

Species	Primer sequence		Probe	
	Forward	Reverse	Reporter	Sequence
F. avenaceum	avenaceumMGBF[1] :CCATCGCCGTGGCTTTC	avenaceumMGBR[1]: CAAGCCCACAGACAC GTTGT	VIC®	avenaceum MGBprobe[1] :ACGCAATTGACTATT GC
F, culmorum	FculC561[2] CACCGTCATTGGTATG TTGTCACT	FculC614[2]: CGGGAGCGTCTGATAG TCG	FAM	FculMGB: TGCTGTCATCACA TTC
F. graminearum	graminearumMGBF[1]: GGCGCTTCTCGTGAA CACA	graminearumMGBR[1]: TGGCTAAACAGCACG AATGC	FAM	graminearumMGBprobe[1]: AGATATGTCTCTTCA AGTCT
F. poae	FpoarA51fwd[2]: ACCGAATCTCAACTCC GCTTT	FpoaeA98rev[2]: GTCTGTCAAGCATGTT AGCACAAGT	VIC®	FpoaeMGB: TGGCGGGGTAGACT
F. sporotrichioides	FspoA18fwd[2]: GCAAGTCGACCACTGT GAGTACA	FspoA85rev[2]: CTGTCAAAGCATGTC AGTAAAAATGAT	VIC®	FsporoMGB: AACCCCGCCCAGACT

§ Waalwijick et al., 2004
† Nicholaisen et al. 2009

Real Time PCR Reactions. Real time PCR was carried out on iQ5 iCycler Real Time detection system (BioRad, Hercules, CA, USA). The SYBR Green reactions were performed using 2x iQ SYBR Green Supermix (50 µL iTaq DNA polymerase, 6 nM $MgCl_2$, dNTPs, SYBR Green I, 20 nM fluorescein) obtained from BioRad, Hercules, CA, USA. A 25 µl reaction contained 12.5 µl of supermix, 6.5 µL sterile nuclease free water (Molecular Biological, Irvine CA, USA), 2 µL of forward primer and 2 µL of reverse primer. 2 µl of template DNA was added to each reaction well, and in negative controls, the template was replaced by 2 µl of sterile nuclease free water. The reactions were carried out in 96 well iQ5 PCR plates (BioRad, Hercules, CA, USA) and sealed with optical sealing tape (BioRad, Hercules, CA, USA). The cycling conditions included a 3 min 95°C step to inactivate any RNA or PCR inhibitors followed by 40 cycles, of 30 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C. After the amplification cycle was completed, the melt curve analysis was done at 55°C to 95°C.

TaqMan® reactions were also carried out using 2x iQ supermix (50 µl iTaq DNA polymerase, 6 nM $MgCl_2$, dNTPs, 20 nM fluorescein). A 25 µl reaction contained 12.5 µl of supermix, 5.5 µl sterile nuclease free water, 2 µl of forward primer and 2 µl of reverse primer and 1 µl of TaqMan® probes. 2 µl of template DNA was added to each reaction well, and in negative controls, the template was replaced by 2 µl of nuclease free water. The template concentration was varied between 50 ng and 0.005 ng. The primer concentration was varied between 1000 nM to 125 nm and TaqMan® probe concentration was varied between 100 nm to 25 nM.

RESULTS AND DISCUSSION

Objective 1: To Compare Three Commercially Available DNA Purification Kits For DNA Yield and Quality

The three kits used in the final DNA extraction experiment were narrowed down from five methods used. The kits evaluated included 4 commercially available DNA isolation kits namely, FastDNA®, Qiagen® DNeasy Blood and Tissue, Qiagen® DNeasy Plant and Wizard® Genomic DNA purification, while a CTAB buffer based protocol, developed by Bluhm et al., 2002, and was also evaluated. The results of the preliminary experiments are given in Table 8.

Table 8: Results from a preliminary assessment of DNA extraction kits

Kit	Yield (ng/μl)	Duration (Hours)	Quality	Cost (100 isolations)
CTAB	10-110	5- 6	Moderate	Low, homemade protocol
Wizard® Genomic DNA purification	55-180	4-5	Low, RNA contamination	$146.00
FastDNA®	202-500	2-3	Good	$236.50 + Homogenizer ($9,900)
Qiagen® DNeasy Blood and Tissue	95-350	4-5	Good	$270.00
Qiagen® DNeasy Plant	65-200	5-5.5	Good	$364.00

The three commercially available DNA isolation kits that showed highest yield and quality were incorporated in the final experimental design. The cetyltrimethylammonium bromide (CTAB) buffer based method was an in-house protocol and the recorded yield was inconsistent. The method was labor intensive with long incubation periods. In view of these limitations, the method was not included in the final experimental design. Similarly, the Wizard® Genomic kit was also eliminated from the final experimental design because of the low DNA yield quality. The DNA appeared chunky and clumped into a mass, which was maybe due to bad DNA quality and improper elution. High RNA concentration was also seen due to the absence of an RNase step in the protocol. Based on the yield range, and duration, the three commercially available DNA isolation kits selected were FastDNA®, Qiagen® DNeasy Blood & Tissue kit and Qiagen® DNeasy Plant kit.

Different lysis methods were also tested for the most effectiveness and efficiency. In one experiment, the samples were flash frozen in liquid nitrogen and ground using a pestle and mortar. Although this method could pulverize the cells well, there were concerns with cross contamination. Use of a tissue grinder to lyse *Fusarium* cells was also examined, but grinding was not adequate. Using enzymatic lysis buffers provided in the commercial DNA isolation kits, proteinase K and heat (65°C) to lyse cells resulted in higher yield for all five species. In subsequent experiments, the method was coupled with mechanical disruption, using FastPrep® 24 Homogenizer, which further increased DNA recovery from the *Fusarium* cells. The data generated from combining enzymatic lysis with mechanical disruption lead to the final experimental design illustrated in Figure 1.

Results of DNA Extraction Experiment. The three commercially available DNA extraction kits selected on the basis of preliminary results (Table 8) were FastDNA®, Qiagen® DNeasy Blood & Tissue and Qiagen® DNeasy Plant. The DNA/Protein ratio (260/280) represents the quality of the DNA isolated. Results showing the yields of the DNA extraction are represented in Figure 2.

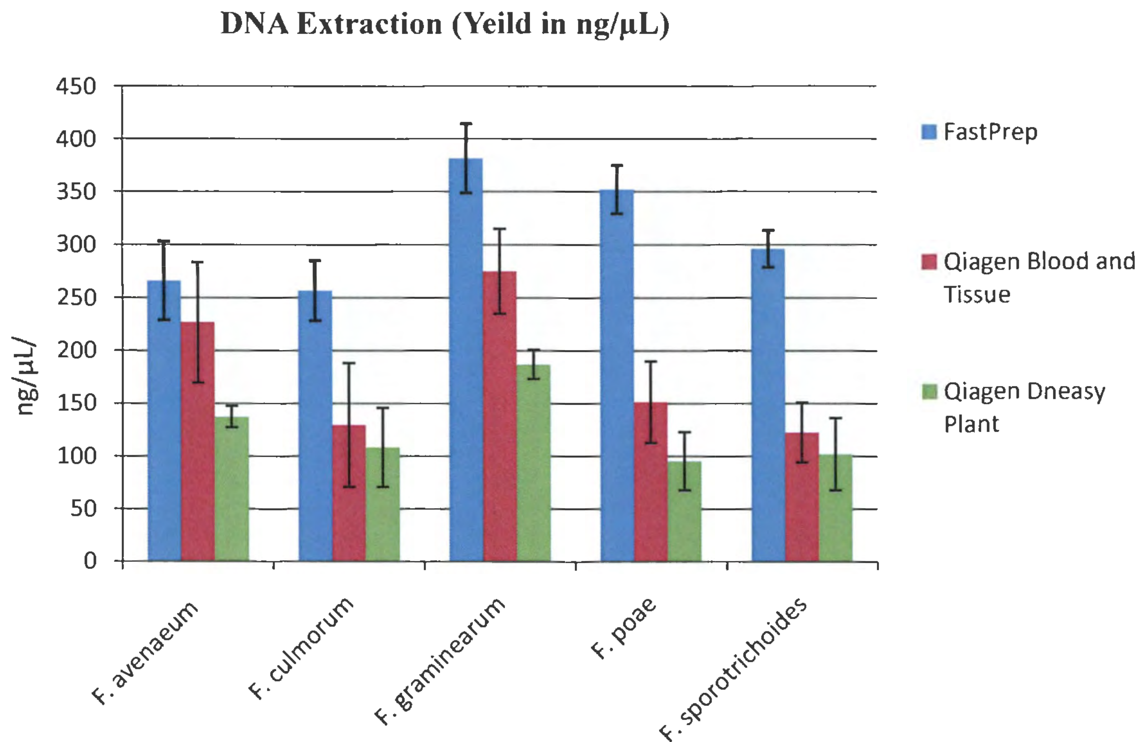

Figure 2: Histogram depicting yield obtained after DNA extraction was performed using three commercially available DNA isolation kits. (Error bars represent the standard deviation).

The FastDNA® extraction kit resulted in the highest yield of DNA and was the most reproducible. The difference in yield between FastDNA and the other two were statistically significant as compared to Qiagen® Blood & Tissue kit ($P < 0.01$), and

Qiagen® Plant kit (P<0.001) for all the *Fusarium* species tested. The only exception was the yield of *F. avenaceum*, for FastDNA and Qiagen Animal in which the P value for was 0.4. This is most likely attributed to the high standard deviation observed in the DNA yield in the Qiagen® Blood & Tissue kit. There was not a significant difference between the Qiagen Blood Tissue kit and Qiagen Animal kit, except in case of *F. culmorum*, *F. poae* and *F. sporotrichioides*. The standard deviations are represented in a graph in Figure 2, while the raw data is shown in Appendix 2. The results of the 2 way ANOVA (Analysis of variance) is represented in Table 9.

Table 9: Results of the two way ANOVA to compare the DNA extraction results between three kits

Organism	FastDNA® vs Qiagen® Animal	FastDNA® vs Qiagen® Plant	Qiagen® Animal vs Qiagen® Plant
F. avenacuem	P > 0.05	P<0.001	P < 0.05
F. culmorum	P<0.001	P<0.001	P > 0.05
F. graminearum	P<0.01	P<0.001	P < 0.05
F. poae	P<0.001	P<0.001	P > 0.05
F. sporotrichioides	P<0.001	P<0.001	P > 0.05

The FastDNA® was found to take less time for isolating DNA (Table8). This is important when these protocols are used for large scale screening of samples for *Fusarium* contamination. The rapid process with fewer steps and high yield will not only reduce the duration, but also decrease chances of cross contamination.

Although Qiagen® DNaeasy Plant kit gave lower yields, the DNA quality seen from the 260/280 and was better as compared to the FastDNA® kit (Table 10). Low ratio

of 260/280 for FastDNA® kit could be due to salt or organic solvent carryover (Fredlund et al. 2008) or difference in the binding method. FastDNA® kit binds the DNA to a silica matrix, while Qiagen® uses a column, (Table 5). This conclusion can be drawn because the three commercially available DNA isolation kits were subjected to the same process for lysis and were provided with same concentrations of proteinase K and RNAse (Figure1).

Table 10: Average ratio of DNA to protein for kits used during DNA extraction			
Species	FastDNA®	Qiagen® DNeasy Blood and tissue	Qiagen® DNeasy Plant
F. avenacuem	1.40	1.80	1.63
F. culmorum	1.27	1.71	1.48
F. graminearum	1.43	1.62	1.48
F. poae	1.58	1.69	1.62
F. sporotrichioides	1.66	1.68	1.52

DNA yield varied between species with *F. graminearum* producing the highest yield, while *F. culmorum* and *F. avenaceum* gave lower yields for 100 mg of wet fungal tissue. This may be due to the cell wall of *F. culmorum* and *F. avenaceum* offering more resistance to lysis. Some studies done on the uptake of heavy metals in *Fusarium* invading cereals showed that *F. avenaceum* and *F. culmorum* are most resistant to the effects of heavy metals, especially Cadmium (Ngu et al., 1997). This suggests that both have a more

impervious cell wall, which resists lysis. Further studies need to be done to understand the complexity and the differences between cell walls of different species of *Fusarium*.

Objective 2: To Develop Individual Real Time Taqman® Probes For Five Species of *Fusarium.*

SYBR Green. The dye was used for detection in the initial qPCR reactions in order to check primer effectiveness and determine the optimum conditions for the primers to function. Initially, primers for all five species were selected from the study by Nicholaisen et al., 2009; however the results were not satisfactory as *F. avenaceum* primers showed cross reactivity, while amplification was not observed for *F. graminearum*, under the altered annealing temperatures. Therefore, only primers for *F. culmorum*, *F. poae* and *F. sporotrichioides* were selected from the study, and primers as well as TaqMan® MGB probes for *F. graminearum* and *F. avenaceum* were selected from a study by Waalwijick et al., 2004. The published cycling conditions were modified to allow the Tm for all five *Fusarium* species to be compatible. The primers and TaqMan® MGB probes from Waalwijick et al., 2004 were designed from RAPD (random amplification of polymorphic DNA) fragments and the primers from Nicholaisen et al., 2009 targeted EF1α region. Primer sets targeting the IGS region of *F. sporotrichioides* were also used in the experiments; however, they showed cross reactivity and were not able to function at the same temperature as the other primers (data not shown). As a preliminary experiment, gradient PCR was done to determine the ideal annealing temperature by testing a range of Tm between 56°C to 60°C, and then an optimum annealing temperature of 58 °C was selected.

The results for amplification and melting curve are represented by Ct and Tm respectively. Figure 3 and 4 show the amplification curve and melt curve for Nicholaisen primers respectively, while Figure 5 and 6 show amplification and melt curves for Waalwijick primers. The Ct values are reported in Table 11 and Table 12 for Nicholaisen and Waalwijick primers respectively. There is some difference between the Ct of replicates 1 and 2 and the Ct from replicate 3. This may be due to some time dependent degradation that may have occurred. The DNA was frozen at -20°C and thawed before each run. This freeze-thawing may have degraded DNA that caused a delayed Ct, which correlates to lower DNA concentration.

Table 11: Ct and Melt Curve values for Nicholaisen primers

Species	Replicate 1			Replicate 2			Replicate 3		
	C_t		T_m	C_t		T_m	C_t		T_m
F. culmorum	15.0	15.0	79.0	15.8	15.3	79.0	18.5	18.5	79.0
F. poae	17.3	17.4	81.5	19.3	19.4	81.5	22.8	22.8	81
F. sporotrichioides	15.2	15.2	82.0	18.8	18.8	82.0	21.2	21.1	82.5

<u>TaqMan® Probes</u>. The TaqMan® MGB probes designed for *F. culmorum*, *F. poae* and *F. sporotrichioides* were tested and the C_t values are reported in Table 13 for varying concentrations of DNA between 50 ng and 0.005 ng. The C_t values for varying the primer pg occurs is 500 nM for *F. avenaceum*, *F. culmorum* and *F. poae*, and is 750 nM for *F. graminearum* and 1 μM for *F. sporotrichioides*. The least probe concentration is 75 nM for all five species for up to 750 nM of primers. The raw data for this experiment are included in Table 24, Appendix 2.

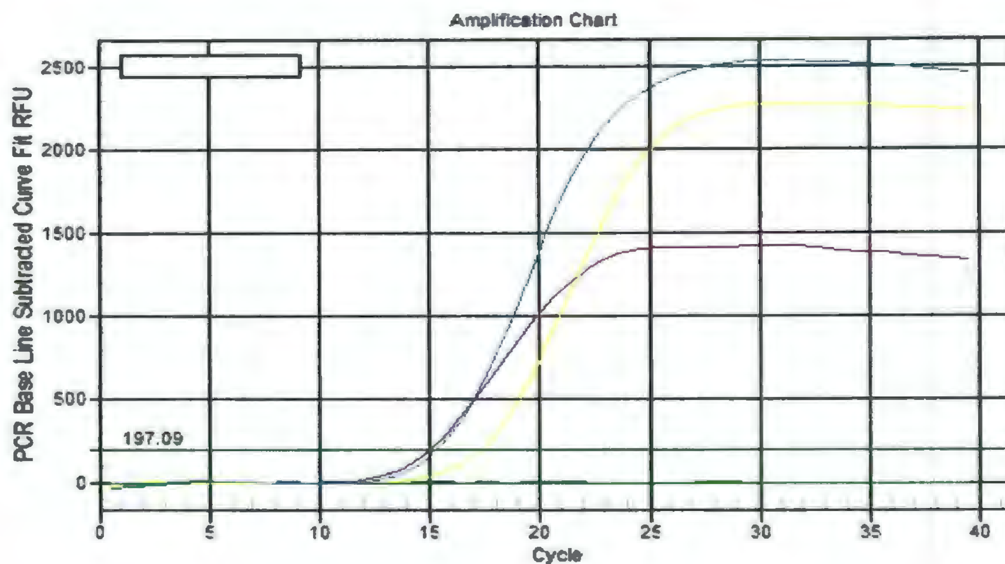

Figure 3: Amplification curves for *F. culmorum* (Pink) ,*F. poae* (Yellow) and *F. sporotrichioides* (Blue) using primers from Nicholaisen et al., 2009

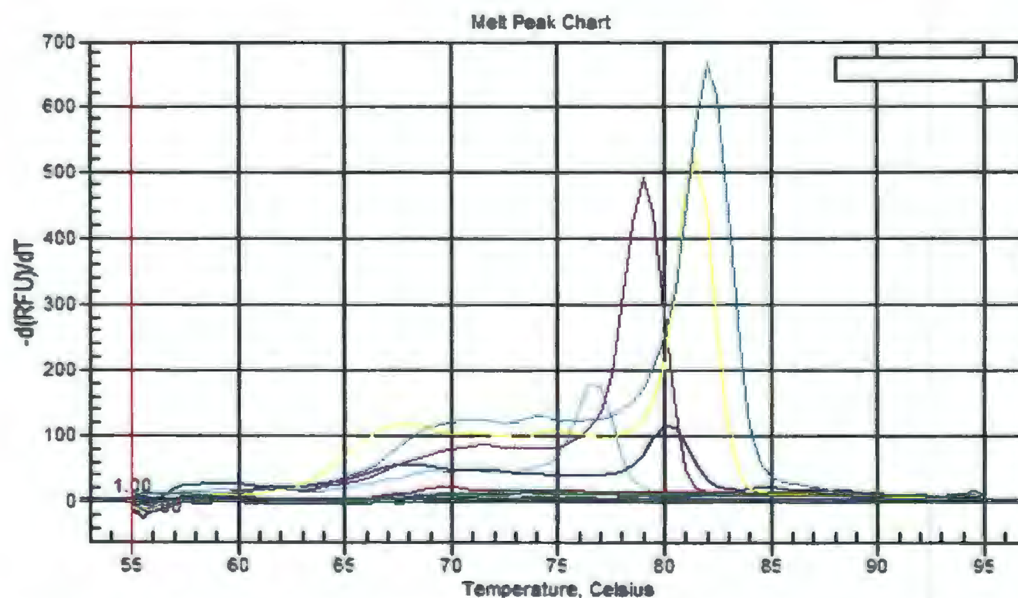

Figure 4: Melting curve for *F. culmorum* (Pink), *F. poae* (Yellow) and *F. sporotrichioides* (Blue) generated by plotting the change in relative fluorescence per unit (ΔRFU)/ unit time (ΔT) against increasing temperature using primers from Nicholaisen et al., 2009

Table 12: SYBR Green Ct and Melt Curve using primers from Waalwijick et al., 2004

Species	Replicate 1			Replicate 2			Replicate 3		
	C_t		T_m	C_t			C_t		T_m
F. avenaceum	15.8	15.2	79.5	15.7	15.1	79.5	15.6	15.9	80.5
F. graminearum	25.8	27.4	84.5	25.7	22.4	84.5	22.4	22.6	84.5

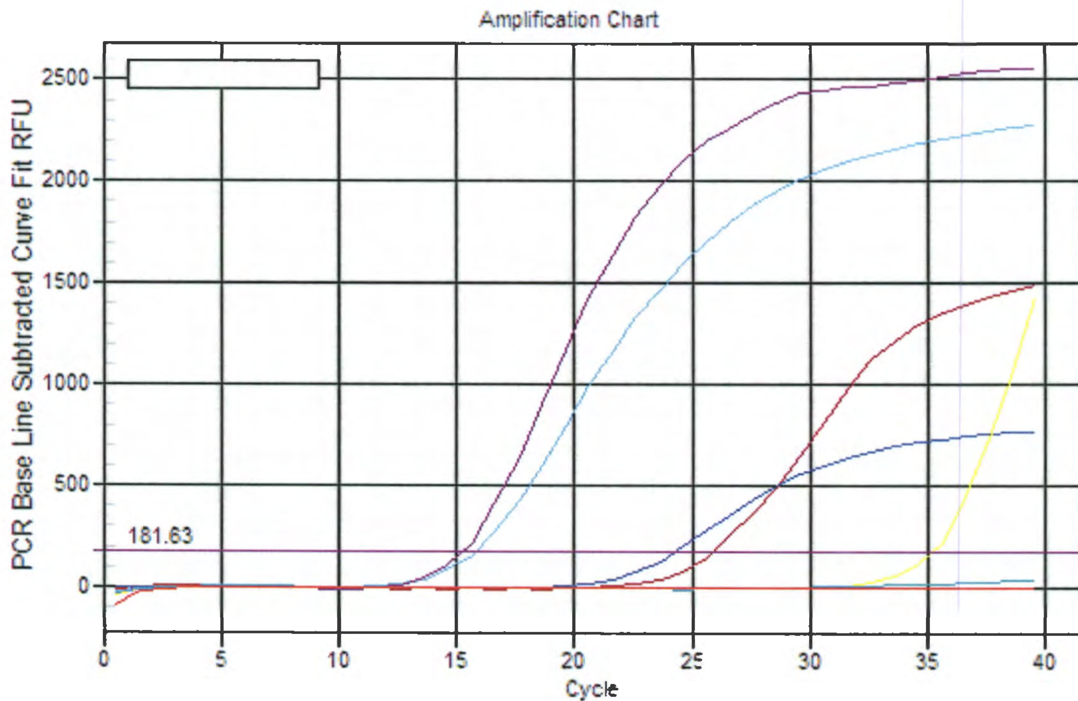

Figure 5`: Amplification curves for *F. avenaceum* (pink and light blue) and *F. graminearum* (red and dark blue) using primers from Waalwijick et al., 2004

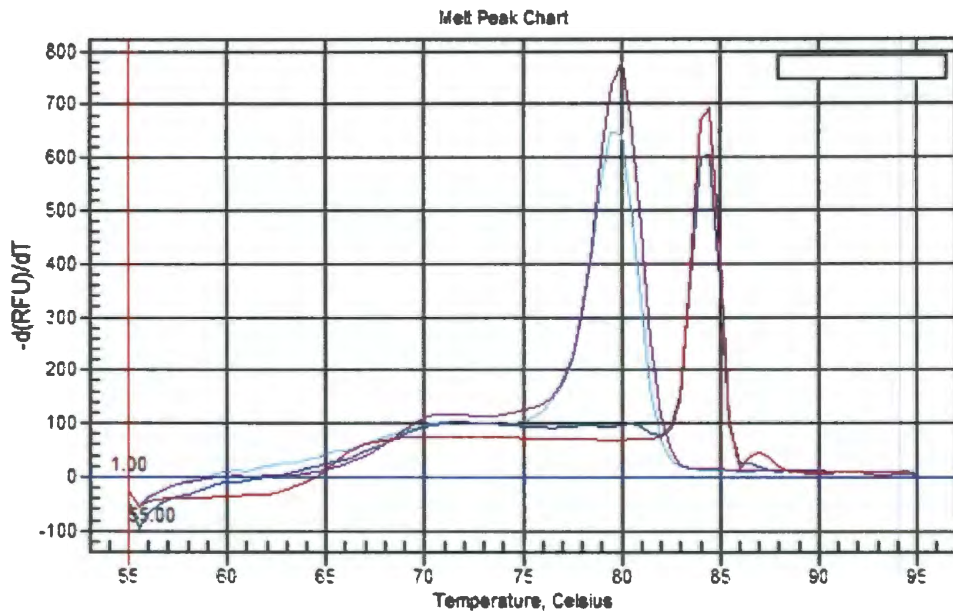

Figure 6: Melting curve for *F. avenaceum* (Pink and light Blue) and *F. graminearum* (Red and Dark Blue) by plotting the change is relative fluorescence per unit (ΔRFU) by the change in temperature (ΔT) using primers from Waalwijick 2004.

Cross reactivity was tested for each species against the other four. Cross reactivity was also checked with *Fusarium acuminatum*, *F. redolens*, *F. proliferatum*, *Aspergillus niger*, *A. ochraeus*, *Penicillium notatum*, *P. vernicossum* and *Geotrchum candidum*, the results for which are described in detail in appendix 2. The cross reactivity tests were negative and all C_t higher than 35 were not included. (Filion et al., 2003, Sarlin et. al. 2006). The correlation coefficient between known quantities of DNA and Ct values (R^2) for TaqMan® MGB probes designed in our study was 0.98 for *F. culmorum* and *F. poae* and 0.978 for *F. sporotrichioides*. A range of primer concentrations were tested for all the species and the experiments indicated that the least primer concentration at which detection of up to 5

Table 13: Ct values for five species by varying template concentration 1 μM primer concentration, and 100 nM probe concentration

Organism	Template concentration (ng)				
	50	5	0.5	0.05	0.005
F. avenaceum	24.2	29.2	29.7	30.3	31.6
F. culmorum	23.2	25.1	29.7	33.2	36.7
F. graminearum	23.5	25.1	28.1	31.2	34.4
F. poae	18.9	22.2	26.6	29.6	33.2
F. sporotrichioides	23.4	25.8	27.2	31.0	34.0

Validation experiments were done to check the functionality of primers and TaqMan® MGB probes in inoculated wheat, the results for which are reported in Table 14. The clean wheat did not show any amplification before the 35th cycle, except in the case of *F. graminearum*, where trace amounts of fungus was detected which could be quantified to less than 5 pg of DNA. Presence of latent infection in the seeds could be a source of this DNA in the clean wheat, as autoclaving would have killed the propagules, but DNA would have persisted.

In experiments with TaqMan® probes, real time PCR was used to detect as well as quantify the amount of fungal DNA, by plotting a standard curve of pure fungal genomic DNA. The data for effectiveness of the assay was validated by inoculating wheat with conidia from five species of *Fusarium*. The fungal infection rate of samples used in Table 14 was 100% and in all cases and they corresponded to ≥ 50 ng of DNA in 2 μl of DNA sample. In terms of number of propagules of *Fusarium*, if one macroconidia corresponds

to 0.03 pg to 0.06 pg of DNA (Filion et al., 2006, Waalwijick et al., 2004), then detection limits of 5 pg obtained from our experiments, corresponds to 84 to 167 macroconidia being detected for every 200 mg of wheat grain. The wheat would contain a mixture of conidia as well as mycelia; hence the sensitivity of this assay is even lower. This shows that the QPCR protocols developed for the five *Fusarium* species is extremely sensitive. In addition, the absence of cross reactivity with any of the strains or species tested indicates the specificity of the assays. The method is rapid, accurate and reliable, and can give a good estimate of possible mycotoxin contamination.

Table 14: Ct from artificially inoculated wheat DNA

Species	Inoculated Wheat C_t					Clean wheat C_t
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	
F. avenaceum	22.1	23.6	21.6	22.4	23.5	36.0
F. culmorum	19.3	22.6	18.1	20.6	21.5	36.6
F. graminearum	18.2	25.3	25.0	19.6	20.0	34.4
F. poae	25.7	23.8	21.5	24.1	24.8	35.8
F. sporotrichioides	19.1	19.3	20.4	20.5	22.3	35.6

The TaqMan® MGB probes method developed acts as an alternative to conventional practices such as culturing, ELISA or conventional PCR. TaqMan® probes also are a valuable tool for risk assessment. The species specific qPCR assays can allow study of individual species to monitor the epidemiology and gain understanding of disease progression using the primers and TaqMan® probes developed in this study. The interaction between environment, host and fungi can be studied by monitoring the

biomass. For example, the recent trend in dominant FHB pathogens indicates a shift from *F. culmorum* to *F. gramiearum* in cooler areas of Eastern Europe. Such changes in pathogen can be easily monitored by performing a large scale screening using qPCR. Evaluation of the prevalent *Fusarium* species can provide a better understanding of disease progression and allow risk assessment and knowledge of prevalent species also helps in monitoring disease spread and devising control measures. These tools can potentially assess the efficacy of control measures and validation of intervention strategies employed can also be addressed. Moreover, in a disease complex such as FHB, the role of individual species is very important, especially because the causal *Fusarium* species are mycotoxigenic with a varied toxin profile and the mycotoxins may interact; showing additive effects and synergism, as seen in outbreaks of ATA, therefore by defining individual organisms, a better understanding of disease complex and potential threats posed by the species involved is achieved.

Overall, we have been successful in designing qPCR based detection and quantification strategies for *F. graminearum, F. culmorum, F. poae, F. sporotrichioides,* and *F. avenaceum* that can be used for detection of these mycotoxigenic species from infected grain samples. The uses of the FastDNA® extraction procedure coupled with qPCR using protocols mentioned, are not only rapid, sensitive and accurate, but can be used for screening multiple samples at one time.

FUTURE RESEARCH

The next step after completion of research objectives would be to validate primer and TaqMan® MGB probe sets from this study on different systems commonly infected with the FHB pathogens, such as barley, maize and oats. The primer optimization need not be repeated but the DNA extractions from the different systems need to be evaluated. The experiments would require an optimized DNA extraction protocol that efficiently extracts DNA from barley and maize, and eliminates PCR inhibitors.

In addition, there have been reported cases of evolutionary shifts in causal species that dominate in the Fusarium head blight disease complex. Monitoring the fungal biomass of five species to map dominant species can be done using the primers and probes from this study. Fusarium head blight is said to spread rapidly and colonize fields overnight (McMullen et al., 1997). Measuring the growth of fungus and quantifying the increase in *Fusarium* biomass at regular intervals reflects the prevalence and disease progression.

Multiplexing can also be achieved once the genomes for each *Fusarium* species get fully sequenced. Presently, three species of Fusarium, namely *F. graminearum*, *F. verticillioides* and *F. oxysporum* have fully sequenced genomes. The sequences available for different regions of *Fusarium* either have limited variability or are not documented for all species. There can be cross reactivity and other issues that may occur when we perform multiplex reactions using primers targeting the same region. After genome sequences are described, designing highly species specific primers can be done. Finally, the primers and probes can also be used to develop multiplex protocols at a later stage. The multiplex

reactions would be able to assay the Fusarium content faster and would be a “single test tube” that detects as well as quantifies the *Fusarium* species

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APPENDIX 1: BACKGROUND INFORMATION ABOUT PCR, qPCR AND PRIMER DESIGN

PCR is becoming a commonly used detection tool because it is rapid and sensitive (Haugland et al., 1999, Mackay I. M., 2004). Two sets of single stranded primers, about 10-25 bases in length that can selectively bind to complementary DNA sequences are used in a PCR. One of the primers is a forward primer that directly forms a stable complex with the 5' to 3' template strand, while the other is a reverse primer that attaches to complimentary sequence in reverse orientation to the anti-parallel 3' to 5' parent DNA. Copies of the region in-between two primers are amplified and the product of amplification is called an amplicon (Konietzny and Greiner, 2003). The PCR process consists of cycles and each cycle is further divided into three steps. The first step is denaturation at 95°C in which the double stranded DNA separates into two parent strands. The separated strands create two sets of templates for the primers to get attached to (Vilijoen et al., 2005). The next step is annealing at 50-60°C, in which the oligonucleotide primers form a duplex with the complimentary DNA sequences. The final step is extension at 70-78°C where DNA polymerase lengthens the strand, generating copies of the targeted portions in the parent strand. The extension of DNA strand requires heat stable polymerase, deoxyneucleotide triphosphate (dNTP), and Mg^{2+} ions to build new DNA strands (Vilijoen et al., 2005) from the parent strands. The number of copies or amplicons generated follows the formula 2^n, where "n" is the number of cycles.

The entire PCR typically comprises 25 to 40 cycles. Using the formula for 30 cycles; $2^{30} = 10.73 \times 10^8$ amplicons should be generated; however the number is lesser due to polymerase inactivation, consumption of reagents or PCR inhibitor accumulation. Before

a reduction in PCR efficiency, the amplification is in the exponential phase where cycle number corresponds to increase in amplicon concentration. After the exponential phase, generally occurring after the 30th cycle, the amplification curve plateaus, and no further increase in amplicon concentration is seen. The PCR products are run on an agarose gel and bands are visualized using a dye that intercalates with DNA. This method, where PCR products are analysed in agarose gel is called an end-point PCR.

qPCR is an advancement over end-point PCR because it allows simultaneous monitoring of the reaction by using a third oligonucleotide, called a probe. The probe is added in addition to the forward and reverse primers used in an end-point PCR. The probe is labeled with fluorescent molecules and it attaches in between the two primers as shown in figure A. The fluorescent molecules report an increase in the concentration of amplicons by emitting fluorescence. The qPCR instrument collects the signals after each cycle and reports them as a plot between the fluorescence vs. cycle number. During the initial cycles, concentration of amplicons is low, thus the fluorescence is not the background. Sufficient increase in concentration of amplicons is seen around the 15th cycle, and the fluorescent signal crosses the background levels. The cycle at which the signal becomes strong enough to be detected by the qPCR instrument is the threshold cycle or C_t. The Ct is inversely related to amount of starting DNA template. For higher concentrations of starting template, low C_t is recorded is because fewer cycles are needed to cross the background fluorescence.

The fluorescent signal measured in a qPCR instrument is generated from two types of DNA binding dyes or fluorophores. The first type is SYBR® Green, which is a nonspecific DNA binding dye. SYBR Green I functions by binding to double stranded

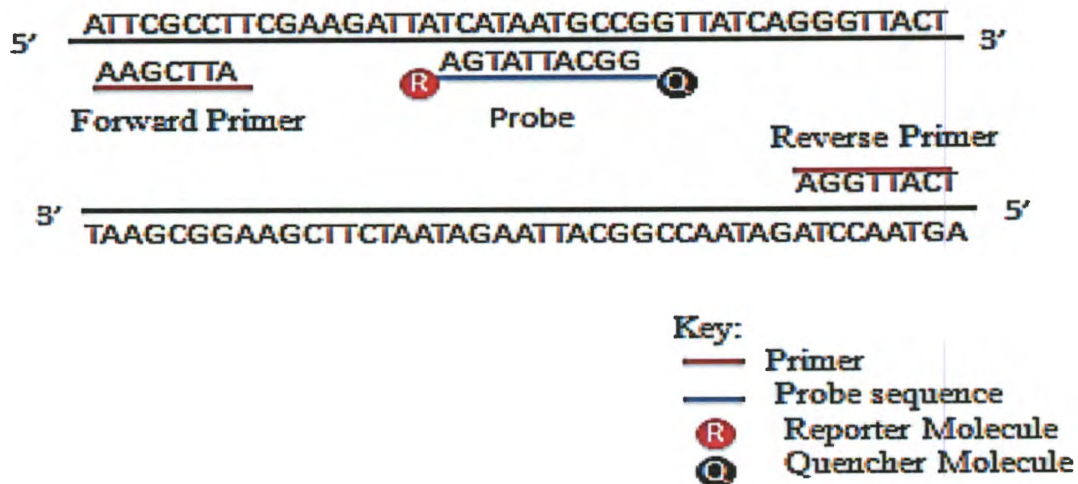

Figure 6: Fluorescent molecules used in PCR:

(ds) amplicons generated after amplification. The second type uses a fluorophore bound to an oligonucleotide which is specific to the template DNA, for example TaqMan®, molecular beacons, or Scorpion® etc. SYBR Green: SYBR Green molecules emit low fluorescence when present freely in the solution, but when bound to dsDNA, the fluorescence increases 1,000 fold (Anonymous, 2006). The fluorescent signal increases with increasing number of cycles because more SYBR Green molecules binds to amplicons. To distinguish and characterize amplified sequences from unwanted PCR products, a melt curve analysis is performed after PCR cycles are complete. The temperature is increased in small increments to denature DNA and dsDNA becomes single stranded (ss). The transformation liberates SYBR Green molecules that were previously bound to ds DNA, lowering the fluorescence. The fluorescent signal after each temperature increment is recorded, and the temperature at which a sudden decrease in fluorescence is observed is documented. A peak is reported by plotting the negative first derivative of relative fluorescence units (-dRFU) against change in temperature (dT). This

peak is the melting temperature (T_m) and is specific for amplicons of defined sequence length. The melting curve peaks distinguish the specific products from non-specific PCR products.

TaqMan®: Is another type of fluorescent molecule used in a QPCR instrument. TaqMan® probe are sequence specific as the fluorescent molecules are linked to oligonucleotides complimentary to the template sequence. The principle for a TaqMan® is based on fluorescence resonance energy transfer (FRET). TaqMan® probes comprise of a signal molecule attached to the oligonucleotide sequence complimentary to template DNA. The probe is flanked by two molecules; a reporter, present at the 5' end and quencher at the 3' end. The reporter molecule emits fluorescence of a particular wavelength, however when present in the vicinity of the quencher the emitted light gets absorbed.

TaqMan® assays are also known as 5' nuclease assay because it uses the 5' activity of *Taq* polymerase to generate fluorescence. The probes hybridize with template DNA after the forward and reverse primers have annealed and then the 5'to 3' exonuclease activity of *Taq* polymerase causes 5' reporter to become liberated. In Free State and the reporter is able to emit fluorescence, which in turn is recorded by filters present in the QPCR instrument, There are a variety of reporter molecules, each having a characteristic excitation and emission wavelength, given in Table 1A. Certain QPCR instruments contain multiple filters to detect different wavelengths from different reporter molecules, enabling the use of different reporter molecules in the same reaction. The choice of fluorescent can be made based on whether the reaction is for single template target (singleplex) or multiple targets (multiplex). A singleplex reaction amplifies a single target

by a set of primers, whereas a multiplex is when more than one locus is amplified in a single reaction using multiple primer and probe sequences (Henagariu et al., 1997). Multiplex assays can be designed by labeling TaqMan® probes having reporters having different emission wavelengths. The excitation and emission wavelengths are mentioned in Table 15.

Apart from detection, QPCR has applications in gene expression or quantification studies, single nucleotide polymorphism (SNP) studies and allele discrimination. QPCR is particularly useful for monitoring the viral load, as viruses cannot be cultured on media. (Anonymous, 2007). The limitation of a TaqMan® assay however is the high cost and complicated design parameters (Anonymous, 2006).

Guidelines for Primer Design

TaqMan® primer design is different from SYBR Green because an additional probe has to be designed for the reaction. A number of primer design software are available for this purpose, including Primer Express 3 (Applied Biosystems, Foster City CA, USA), Primer 3 (Massachusetts Institute of technology, USA), PrimeTime® from Integrated DNA Technologies (Corallville, IA, USA) and Beacon Designer. The length of amplicon should be between 70 and 200 base pairs (bp) long (Boeckman et. al) and possibility of secondary structures like hairpin loops and primer-dimers should be minimized. The guanidine to cytosine (GC) content in primers should lie between 20% and 80% (Boeckman et al., Hyndman et al., 2003, Anonymous, 2006) and the primer annealing temperature should lie between 56-60°C. The primer sequence should be free of repetitions of a single nucleotide (Boeckman et al.).

Table 15: Excitation and emission wavelength of commonly used QPCR fluorophores and quenchers

Filter (BioRad iQ5)	Fluorophore	Excitation (nM)	Emission (nM)	Quenchers
II	6-Carboxy FAM	492	516	Black Hole Quencher (BHQ) 1, 6-carboxytetramethylrhodamine (TAMRA), Minor Groove Binder (MGB)
	SYBR Green I	492	516	-
III	TET	517	538	BHQ1, MGB
	HEX	535	555	BHQ1, BHQ2, MGB
	VIC®	535	555	BHQ2, MGB
IV	Cy3	545	568	BHQ2
	TAMRA®	556	580	BHQ2
V	ROX	585	610	BHQ2, BHQ3
	Texas Red	585	610	BHQ2, BHQ3
VI	Cy5	635	665	BHQ2, BHQ3

The probe temperature should be 8-10°C higher than the primers so that primers anneal before the probes. The reporter-quencher combination should be carefully selected, because not all quenchers quench the reporter molecules. The possible combinations are given in Table 14. A quencher moiety, known as minor groove binder (MGB) was developed for TaqMan® assays to enhance probe binding to DNA sequence. Hybridization of MGB with the single stranded DNA molecule results in an extremely stable complexes because MGB places itself in between the minor grooves on the DNA strand, using Van

der Waal forces. In addition, the modified probes containing the MGB show increased annealing temperature by 15-30°C (Anonymous, 2002, Kutyavin et al., 2000)

APPENDIX 2: RAW DATA FOR DNA EXTRACTION AND PROBES

Table 16: DNA extraction data from *F. avenaceum*

Extraction Kit	Replicate	Yield (ng/µl)	260/280	260/230*
FastPrep®				
	1	278.0	1.18	0.39
	2	311.2	1.26	0.43
	3	252.1	1.49	0.39
	4	293.2	1.44	1.3
	5	204.6	1.64	0.28
	6	202	1.55	0.43
Qiagen® DNeasy Blood and Tissue	1	275.3	1.94	1.23
	2	321.2	1.81	0.99
	3	273.3	1.68	0.97
	4	168.9	1.82	1.26
	5	180.4	1.73	0.95
	6	142.4	1.82	0.98
Qiagen® DNeasy Plant	1	132.7	1.69	0.97
	2	137.2	1.75	0.93
	3	128.3	1.47	0.81
	4	140.5	1.68	1.01
	5	131.2	1.58	0.64
	6	156.6	1.61	0.74

*260/230 measures the contaminants like degraded oligos, phenolates or any other small compounds

Table 17: DNA extraction results for *F. culmorum*

Extraction Kit	Replicate	Yield (ng/µl)	260/280	260/230
FastPrep®	1	306.5	1.29	0.23
	2	257.5	1.43	0.28
	3	270.7	1.29	8.92
	4	212.2	1.17	1.04
	5	306.5	1.22	0.23
	6	244.0	1.23	0.24
Qiagen® DNeasy Blood and Tissue	1	115.8	1.97	0.87
	2	164.7	1.57	0.97
	3	136.4	1.2	-
	4	111.2	1.77	-
	5	130.6	1.32	0.97
	6	119.8	1.26	-
Qiagen® DNeasy Plant	1	105.1	1.31	0.84
	2	140.4	1.61	1.16
	3	73.9	1.57	0.88
	4	54.45	1.56	0.88
	5	136.3	1.42	0.87
	6	142.0	1.4	0.88

Table 18: DNA extraction results for *F. graminearum*				
Extraction Kit	Replicate	Yield (ng/µl)	260/280	260/230
FastPrep®	1	451.5	1.25	0.23
	2	397.7	1.15	0.67
	3	275.0	1.64	0.42
	4	387.9	1.52	0.67
	5	447.0	1.46	0.78
	6	332.7	1.54	0.58
Qiagen® DNeasy Blood and Tissue	1	320.8	1.64	0.42
	2	347.9	1.68	0.87
	3	229.7	1.74	0.83
	4	265.3	1.55	0.73
	5	218.4	1.33	0.97
	6	268.8	1.76	0.80
Qiagen® DNeasy Plant	1	236.5	1.52	1.14
	2	287.1	1.55	1.14
	3	164.1	1.48	0.68
	4	134.4	1.53	0.81
	5	159.3	1.44	0.63
	6	143.3	1.36	0.72

Table 19: DNA extraction results for *F. poae*				
Extraction Kit	Replicate	Yield (ng/μl)	260/280	260/230
FastPrep®	1	331.1	1.18	0.67
	2	356.0	1.14	0.77
	3	295.5	1.52	0.67
	4	345.4	1.5	0.63
	5	331.1	1.18	0.11
	6	456.0	1.14	0.77
Qiagen® DNeasy Blood and Tissue	1	219.1	1.71	0.53
	2	136.0	1.76	0.58
	3	119.5	1.69	0.53
	4	113.8	1.31	0.76
	5	155.1	1.38	0.97
	6	166.5	1.68	0.57
Qiagen® DNeasy Plant	1	109.5	1.69	0.89
	2	127.7	1.6	0.97
	3	117.7	1.57	0.96
	4	91.4	1.6	0.97
	5	62.1	1.71	0.75
	6	64.7	1.57	0.7

Table 20: DNA extraction results for *F. sporotrichioides*

Extraction Kit	Replicate	Yield (ng/μl)	260/280	260/230
FastPrep®	1	270.8	1.38	2.22
	2	263.9	1.84	0.24
	3	263.9	1.84	2.22
	4	227.5	1.78	0.24
	5	248.1	1.61	0.98
	6	504.0	1.3	0.34
Qiagen® DNeasy Blood and Tissue	1	90.6	1.84	0.72
	2	127.5	1.83	0.79
	3	131.4	1.57	0.76
	4	145.9	1.56	0.97
	5	131.4	1.75	0.69
	6	109.3	1.57	0.84
Qiagen® DNeasy Plant	1	143.3	1.52	0.85
	2	165.8	1.54	0.97
	3	65.5	1.43	0.8
	4	43.2	1.67	0.88
	5	103.45	1.21	1.41
	6	92.5	1.75	1.22

Table 21: DNA extraction yields (used in Figure 2)			
Species	FastDNA® (ng/µl)	Qiagen® DNeasy Blood & Tissue (ng/µl)	Qiagen® DNeasy Plant (ng/µl)
F. avenacuem	266.2 ± 37.2	227.0 ± 56.9	137.7 ± 10.2
F. culmorum	256.9 ± 28.5	129.8 ± 58.7	108.7 ± 37.5
F. graminearum	382.0 ± 32.9	275.2 ± 40.1	187.5 ± 13.8
F. poae	352.5 ± 22.8	151.7 ± 38.7	95.5 ± 27.6
F. sporotrichioides	296.4 ± 17.4	122.7 ± 28.5	102.3 ± 34.2

Table 22: Cross reactivity against species of *Fusarium*					
Organism	Primers				
	F. avenaceum	*F culmorum*	*F. graminearum*	*F. poae*	*F. sporotrichioi des*
F. avenaceum	+	-	–	–	–
F. culmorum	–	+	–	–	–
F. gramiearum	–	–	+	–	–
F. poae	–	–	–	+	–
F. sporotrichioides	–	–	–	–	+
F. redolens	–	–	–	–	–
F. acuminatum	–	–	–	–	–
F. proliferatum	–	–	–	–	–
Aspergillus ochraceus	–	–	–	–	–
A. niger	–	–	–	–	–
P. vernucossum	–	–	–	–	–
P. chrysogenum	–	–	–	–	–

Table 23: GenBank accession number and the strain information		
Organism	GenBank Accession Number	Gene Target
F. avenaceum	EU220414	*Gibberella avenacea* isolate UR04 elongation factor 1 alphha gene
	EF512021	*Gibberella avenacea* strain IBT9581 translation elongation factor 1 alpha (tef-1alpha) gene
	EF512020	*Gibberella avenacea* strain FRL559 translation elongation factor 1 alpha (tef-1alpha) gene
	EF512019	*Gibberella avenacea* strain DAOM238690 translation elongation factor 1 alpha (tef-1alpha) gene
	EF512018	*Gibberella avenacea* strain DAOM238691 translation elongation factor 1 alpha (tef-1alpha) gene
	EF105293	*Gibberella avenacea* translation elongation factor 1 alpha (tef-1alpha) gene
F. culmorum	EU220412	*Fusarium culmorum* isolate IPO-39 elongation factor 1 alpha gene
	EU220410	*Fusarium culmorum* isolate NL110-1 elongation factor 1 alpha gene
	FJ939672	*Fusarium culmorum* isolate 26 translation elongation factor 1 alpha gene
	FJ939671	*Fusarium culmorum* isolate 653 translation elongation factor 1 alpha gene
	FJ939670	*Fusarium culmorum* isolate 164 translation elongation factor 1 alpha gene
	FJ939669	*Fusarium culmorum* isolate 142 translation elongation factor 1 alpha gene
	FJ939668	*Fusarium culmorum* isolate 141 translation elongation factor 1 alpha gene
	FJ939667	*Fusarium culmorum* isolate 131 translation elongation factor 1 alpha gene
	FJ939666	*Fusarium culmorum* isolate 92 translation elongation factor 1 alpha gene
	FJ939665	*Fusarium culmorum* isolate 19 translation elongation factor 1 alpha gene
	FJ939664	*Fusarium culmorum* isolate 01 translation elongation factor 1 alpha gene
	FJ939663	*Fusarium culmorum* isolate 646 translation elongation factor 1 alpha gene
	EU220410	*Fusarium culmorum* isolate NL110-1 elongation factor 1 alpha gene

(Continued)		
F. graminearum	EF428714	*Gibberella zeae* strain NRRL 34587 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428702	*Gibberella zeae* strain NRRL 34524 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428701	*Gibberella zeae* strain NRRL 34522 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428700	*Gibberella zeae* strain NRRL 34521 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428699	*Gibberella zeae* strain NRRL 34519 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428698	*Gibberella zeae* strain NRRL 34516 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428697	*Gibberella zeae* strain NRRL 34515 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428696	*Gibberella zeae* strain NRRL 34500 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF428695	*Gibberella zeae* strain NRRL 34499 translation elongation factor 1 alpha gene, exons 2 through 4 and partial cds
	EF521161	*Gibberella zeae* isolate DAOM194276 translation elongation factor-1 alpha gene, partial sequence
	EF521160	*Gibberella zeae* isolate DAOM194192 translation elongation factor-1 alpha gene,
	EF521159	*Gibberella zeae* isolate DAOM238708 translation elongation factor-1 alpha gene
F. poae	EU744836	*Fusarium poae* strain I14-99 translation elongation factor 1 alpha gene, partial cds
	EU744835	*Fusarium poae* strain 4/4343/1 translation elongation factor 1 alpha gene, partial cds
	EU744834	*Fusarium poae* strain 141 translation elongation factor 1 alpha gene, partial cds
	EU744833	*Fusarium poae* strain 173 translation elongation factor 1 alpha gene, partial cds
	EU744832	*Fusarium poae* strain 295 translation elongation factor 1 alpha gene, partial cds

(Continued)		
	EU744831.1	*Fusarium poae* strain 300 translation elongation factor 1 alpha gene, partial cds
	EU744830	*Fusarium poae* strain 301 translation elongation factor 1 alpha gene, partial cds
	EU744829	*Fusarium poae* strain 504 translation elongation factor 1 alpha gene, partial cds
	EU744828	*Fusarium poae* strain 507 translation elongation factor 1 alpha gene, partial cds
	EU744827	*Fusarium poae* strain 747 translation elongation factor 1 alpha gene, partial cds
	EU744826	*Fusarium poae* strain 303 translation elongation factor 1 alpha gene, partial cds
	EU744825	*Fusarium poae* strain 444 translation elongation factor 1 alpha gene, partial cds
	EU744824	*Fusarium poae* strain 506 translation elongation factor 1 alpha gene, partial cds
	EU744822	*Fusarium poae* strain 552 translation elongation factor 1 alpha gene, partial cds
	EU744821	*Fusarium poae* strain 563 translation elongation factor 1 alpha gene, partial cds
F. sporotrichioides	AY337442	*Fusarium sporotrichioides* strain FRC T0572 translation elongation factor EF1 alpha gene, partial cds
	AY337442	*Fusarium sporotrichioides* strain FRC T0572 translation elongation factor EF1 alpha gene, partial cds
	EF521146	*Fusarium sporotrichioides* isolate DAOM194202 translation elongation factor-1 alpha gene, partial sequence
	AY337442	*Fusarium sporotrichioides* strain FRC T0572 translation elongation factor EF1 alpha gene, partial cds
	EF521145	*Fusarium sporotrichioides* isolate DAOM194204 translation elongation factor-1 alpha gene, partial sequence

Table 24: Ct values after varying the primer concentration.
(The minimum primer concentration for detection is represented with a bold font)

Primer conc. (nM)	Template DNA	*F. avenaceum*	*F. culmorum*	*F. graminearum*	*F. poae*	*F. sporotrichioides*
1000	**50**	24.21	22.32	23.54	20.05	**23.37**
	5	29.21	25.07	25.14	23.08	**25.78**
	0.5	29.74	29.74	28.06	28.4	**27.22**
	0.05	30.35	33.15	31.25	31.72	**31.05**
	0.005	31.65	36	34.39	34.32	**34.05**
750	**50**	24.58	22.51	**23.62**	20.91	25.76
	5	29.15	25.8	**25.45**	24.88	30.63
	0.5	33.35	30.05	**28.72**	29.55	N/A
	0.05	31.78	33.4	**33.01**	32.55	N/A
	0.005	33.8	36.68	**35.59**	35.37	N/A
500	**50**	**28.31**	**23.19**	24.29	**21.03**	N/A
	5	**32.84**	**27.4**	25.94	**25.99**	N/A
	0.5	**34.35**	**30.61**	29.44	**30.07**	N/A
	0.05	**34.84**	**36.25**	N/A	**33.43**	N/A
	0.005	**35.35**	**38**	N/A	**34.58**	N/A
250	**50**	28.83	29.12	N/A	N/A	N/A
	5	36.31	31.42	N/A	N/A	N/A
	0.5	34.35	N/A	N/A	N/A	N/A
	0.05	N/A	N/A	N/A	N/A	N/A
	0.005	N/A	N/A	N/A	N/A	N/A
125	**50**	N/A	N/A	N/A	N/A	N/A
	5	N/A	N/A	N/A	N/A	N/A
	0.5	N/A	N/A	N/A	N/A	N/A
	0.05	N/A	N/A	N/A	N/A	N/A
	0.005	N/A	N/A	N/A	N/A	N/A

APPENDIX 3: PRELIMINARY EXPERIMENT TO EVALUATE EFFICACY OF TAQMAN® PRIMER-PROBE SETS DEVELOPED IN THE STUDY FOR USE IN DUPLEX qPCR REACTIONS

The primer and TaqMan® MGB probes developed in our study were evaluated for the possibility of duplexing. The *F. culmorum* probe was labeled with FAM, while *F. avenaceum, F. poae* and *F. sporotrichioides* was labeled with VIC®. *F. culmorum* (FC) is duplexed with *F. avenaceum* (FA), *F. poae* (FP) and *F. sporotrichioides* (FS). FAM was detected by filter 2 while VIC® is detected by filter 3 of the BioRad iQ5 iCycler. The reaction was performed for DNA template concentration of 50 ng to 0.005 ng and primer concentration of 1000 nM, 750 nM and 500 nM for FC/FA and FC/FP and 1000nM for FC/FS combination.

For TaqMan® reactions performed in duplexes, the supermix used was iQ Multiplex Powermix (50 µL iTaq DNA polymerase, 11 nM $MgCl_2$, dNTPs, 20 nM fluorescein) from BioRad, Hercules, CA, USA. A 25 µL reaction contained 12.5 µL of supermix, 5.5 µL sterile nuclease free water. 1 µL each of forward and reverse primer from one species and 1 µL each of forward and reverse primer from the other species was added to the reaction. The reactions were carried out in 96 well iQ5 PCR plates (BioRad, Hercules, CA, USA) and sealed with optical sealing tape (BioRad, Hercules, CA, USA). The thermal cycling conditions for the TaqMan® reactions for both single and duplexes included a 3 min 95°C step to inactivate RNA or PCR inhibitors. The next step was 40 cycles, monitored in real-time and consisted of 30 sec at 95°C, 1 min at 58°C and 30 sec at 72°C.

Quantification of unknown quantities of DNA was done by standard curves generated using 10 fold serial dilutions of 50×10^{-9}g to 5×10^{-12}g of genomic DNA from pure cultures. Genomic DNA extracted from wheat samples were diluted 1:10 dilution before PCR (Nicholaisen et al., 2008). The results amplification curves (Figure 8, Figure 9 and Figure 10) showed that the RFU plotted on the Y axis values were not high. This means that the fluorescent signals generated from the probes during duplexes were not beyond the background.

Table 25: Result of duplexes with *Fusarium avenaceum* (FA) and *Fusarium culmorum* (FC)

Flurophore	Sample	Template concentration (ng)	C_t
FAM	Fc (+ control)	50	22.63
FAM	FA/FC	50	26.95
FAM	FA/FC	5	29.65
FAM	FA/FC	0.5	31.07
FAM	FA/FC	0.05	32.25
FAM	FA/FC	0.005	N/A
VIC	Fa (+ control)	50	24.67
VIC	FA/FC	50	25.93
VIC	FA/FC	5	29.45
VIC	FA/FC	0.5	33.19
VIC	FA/FC	0.05	33.96
VIC	FA/FC	0.005	36.74

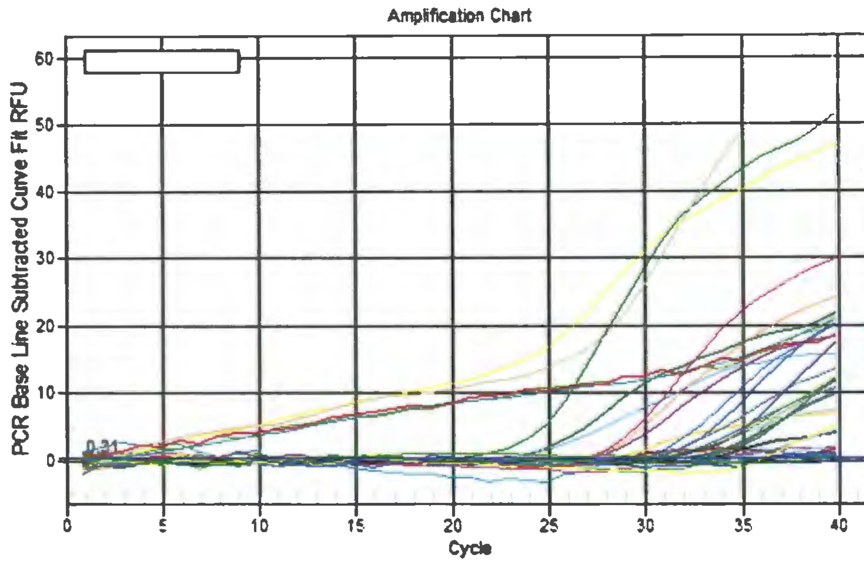

Figure 7: Duplex qPCR reactions for *F. avenaceum* (FA) and *F. culmorum* (FC).

Table 26: Duplex qPCR data for *F. culmorum* (FC) and *F. poae* (FP)			
Flurophore	Sample	Template concentration (ng)	C_t
FAM	Fc (+ control)	50	24.63
FAM	FA/FC	50	27.20
FAM	FA/FC	5	30.95
FAM	FA/FC	0.5	33.15
FAM	FA/FC	0.05	N/A
FAM	FA/FC	0.005	N/A
VIC	FP (+ control)	50	17.98
VIC	FP/FC	50	19.06
VIC	FP/FC	5	24.39
VIC	FP/FC	0.5	27.02
VIC	FP/FC	0.05	N/A
VIC	FP/FC	0.005	N/A

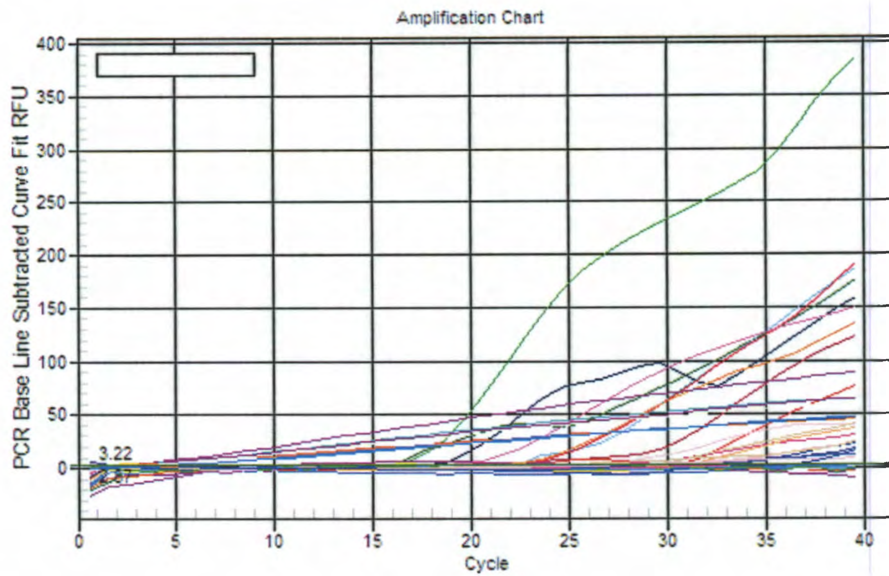

Figure 8: Graph for duplex qPCR reactions of *F. culmorum* and *F. poae*

Table 27: Duplex qPCR data for *F. culmorum* and *F. sporotrichioides*			
Flurophore	Sample	Template concentration (ng)	C_t
FAM	Fc (+ control)	50	25.03
FAM	FA/FC	50	27.20
FAM	FA/FC	5	30.95
FAM	FA/FC	0.5	33.15
FAM	FA/FC	0.05	34.85
FAM	FA/FC	0.005	N/A
HEX	FS (+ control)	50	17.98
HEX	FS/FC	50	20.86
HEX	FS/FC	5	28.57
HEX	FS/FC	0.5	30.02
HEX	FS/FC	0.05	34.03
HEX	FS/FC	0.005	N/A

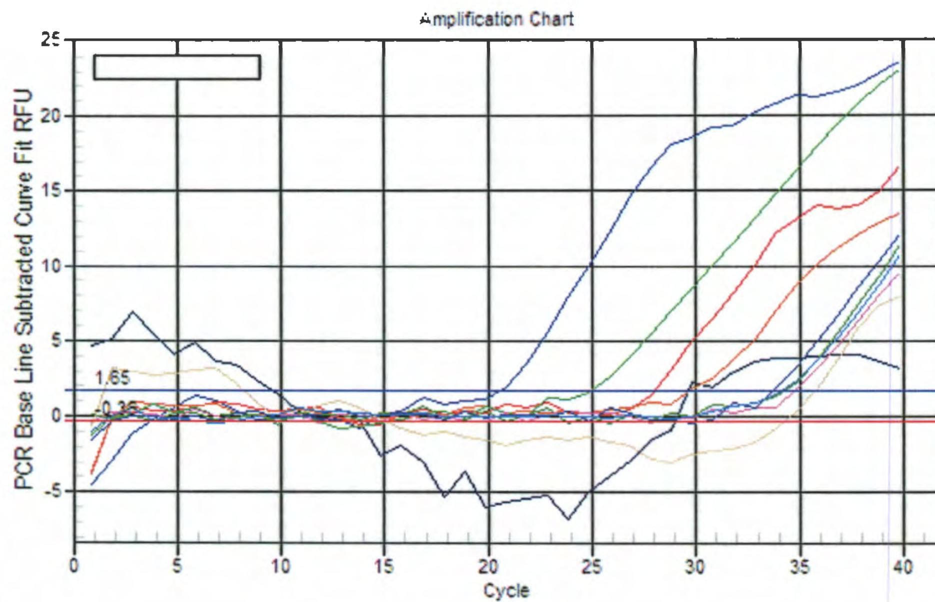

Figure 9: Duplex qPCR of *F. culmorum* (FC) and *F. sporotrichioides* (FS)

The signal strength for RFU seen on the Y axis of the amplification curves was low for FC/FA and FC/FP duplexes. The low signal strength can also be background noise, and not amplification. This can be explained by the quenching action that nucleotide G shows.

F. culmorum Ct was higher than that of *F. poae*, which resulted in indistinct quantification curves. The reproducibility of duplexes was inconsistent, and the Ct values differed by 2 to 3 cycles for some reactions. This could be to competitive inhibition between primers. In some reactions, an early Ct of 3.10 to 5.58 was seen (data not shown), which could be attributed to the probes getting cleaved early and giving out fluorescent signals in the form of straight lines instead of curves. The amplification curves were also not as smooth as seen in singleplexes and did not function at concentrations lower than 750 nM. For multiplex reactions the TaqMan® primers and probes should be compatible

and have comparable Tm. Since we used pre-existing primers and designed our TaqMan® probes around them, the results may have been compromised. By designing primers and probes together

Preliminary results showed that although duplex/multiplex PCR is a good tool for detection and quantification, it may not be as reliable or reproducible as compared to singleplex reactions. TaqMan® assays done using singleplexes give lower C_t values, detecting DNA sooner and more efficiently. A 96 well plate, run with single set of primers and probes at the same annealing temperature gave more reliable results as compared to duplexes. The singleplexes were also able to function at a much lower concentration as compared to duplexes that had limited detection capabilities at lower primer or probe concentration. The difference in duplexing results was probably due to competitive inhibition between the primers or interaction between the templates, primers or probes. To overcome the mentioned limitations we can increase the amount reaction mixture added from 25 μl to 50 μl, however it is not a cost effective alternative to the singleplex reactions that were comparatively more functional at 25 μl.