USDA-ARS | U.S. Wheat and Barley Scab Initiative

FY21 FINAL Performance Progress Report

Due date: July 26, 2023

Cover Page

USDA-ARS Agreement ID:	59-0206-1-201
USDA-ARS Agreement Title:	Management and Innovative Research of FHB in Barley
Principle Investigator (PI):	Thomas Baldwin
Institution:	North Dakota State University
Institution UEI:	EZ4WPGRE1RD5
Fiscal Year:	2021
FY21 USDA-ARS Award Amount:	$24,718
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Period of Performance:	6/01/21 -5/31/23
Reporting Period End Date:	5/31/2023

USWBSI Individual Project(s)

USWBSI Research Category‡	Project Title	ARS Award Amount
BAR-CP	Coordinated Fungal Biomass Measurements of FHB in Barley and Microbial Fingerprinting	$24,718
	FY21 Total ARS Award Amount	$24,718

I am submitting this report as a: ☒ FINAL Report

I certify to the best of my knowledge and belief that this report is correct and complete for performance of activities for the purposes set forth in the award documents.

Thomas T. Baldwin

Principal Investigator Signature

7/26/2023

Date Report Submitted

‡ BAR-CP – Barley Coordinated Project
DUR-CP – Durum Coordinated Project
EC-HQ – Executive Committee-Headquarters
FST-R – Food Safety & Toxicology (Research)
FST-S – Food Safety & Toxicology (Service)
GDER – Gene Discovery & Engineering Resistance
HWW-CP – Hard Winter Wheat Coordinated Project
MGMT – FHB Management
MGMT-IM – FHB Management – Integrated Management Coordinated Project
PBG – Pathogen Biology & Genetics
TSCI – Transformational Science
VDHR – Variety Development & Uniform Nurseries
NWW –Northern Soft Winter Wheat Region
SPR – Spring Wheat Region
SWW – Southern Soft Red Winter Wheat Region

Project 1: Coordinated Fungal Biomass Measurements of FHB in Barley and Microbial Fingerprinting

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1. What are the major goals and objectives of the research project?

The use of hydrolysis probes offers multiplexing options. The main goal of this project was to develop and validat a protocol to quantify *F. graminearum* biomass using Taqman multiplex qPCR. We designed primers and probes specific to *Tri5* and *actin* genes. The *Tri5* gene is common to DON-producing Fusarium species, particularly *F. graminearum* (Hohn and Beremand, 1989), while actin plays essential roles in cell shape, cell division, and mobility (Ruan et al., 2007; Stürzenbaum et al., 2001). Additionally, we developed a protocol for utilizing a 384-well capacity real-time PCR to allow high-throughput assays. The multiplex qPCR protocol we developed and validated enables high-throughput, reliable, and sensitive detection of *F. graminearum* biomass in barley spikes and grains.

2. What was accomplished under these goals or objectives? *(For each major goal/objective, address these three items below.)*

a) What were the major activities?

1. Development and validation of Taqman qPCR primers

The *Tri5* sequence was accessed from *F. graminearum* reference strain PH1 (FGRAMPH1_01G13111) in FungiDB (Basenko et al., 2018). The *actin* sequence was retrieved from Gines et al. (2018) and had NCBI Accession No. AY145451. We designed the primers and probes for *Tri5* and *actin* using the Primer3 software (Koressaar, T. and Remm, M., 2007. Untergasser et al., 2012; Kõressaar et al., 2018) and sequences are shown in Table 1.

Table 1. List of primers and probes used in this study.

Primer/ Probe[a]	Primer sequence (5'–3')	Target Gene
TRI5QPF2	CTCACCCAGGAAACCCTACA	*Tri5*
TRI5QPP2[b]	[FAM]-GATGGTTGCTGTCTTCTCGG-[BHQ1a-Q]	
TRI5QPr2	CATCACCTGAGGGTCCTTGT	
ActinQPF2	CCAGGTATCGCTGACCGTAT	*actin*
ActinQPP2[c]	[TxRed]-GAAGATCAAGGTCGTCGCTC-[BHQ2a-Q]	
ActinQPR2	GCTGAGTGAGGCTAGGATGG	

Assay Development and Optimization. Using gradient PCR, we identified 58°C as the best annealing temperature for *Tri5* and *actin* genes based on lower *Cq* values than higher temperatures and the non-amplification of no template control (NTC). The optimized qPCR profile consisted of a denaturation step of 95°C for 5:00 min followed by 35 cycles of 95°C for 0:15 s and 58°C for 30 s. At this condition, *Tri5* and *actin* genes detection yielded PCR efficiencies (%E) of 103.9 and 93.8% for single runs and 101.5 and 106.2% for multiplex runs,

respectively (Table 2). The %E of singleplex and multiplex runs are within the ideal PCR efficiency range from 90 to 110%. The R^2 for all reactions is above 0.99. The *Cq* values from the tests of the two operators did not vary and were combined for analysis. Singleplex and multiplex reactions at varying concentrations have similar *Cq* values, indicating no inhibition for detecting both genes in the same reaction mixture.

Table 2. Comparison of *Cq* values from singleplex and multiplex reactions of *Tri5* and *actin* genes at varying concentrations.

Concentration (ng/ul)		*Tri5*			*actin*		
Tri5	*actin*	Singleplex[a]	Multiplex[ab]	*P*-value[c]	Singleplex[a]	Multiplex[ab]	*P*-value[c]
30	300	16.3 ± 1.0	16.7 ± 0.5	0.77	20.5 ± 0.8	21.1 ± 1.0	0.70
3	30	19.8 ± 0.8	20.2 ± 0.7	0.63	24.1 ± 0.5	24.0 ± 1.3	0.56
0.3	3	23.1 ± 0.8	23.4 ± 0.7	0.58	27.5 ± 0.9	27.1 ± 1.3	0.99
0.03	0.3	26.3 ± 1.2	26.6 ± 1.0	0.71	31.1 ± 1.0	30.8 ± 0.8	0.11
%E		*103.9*	*101.5*	*N/A*	*93.8*	*106.2*	*N/A*
R^2		*1.000*	*1.000*	*N/A*	*0.999*	*0.994*	*N/A*
Slope		*-3.232*	*-3.286*	*N/A*	*-3.510*	*-3.180*	*N/A*
y-int		*21.51*	*22.91*	*N/A*	*29.33*	*27.88*	*N/A*

[a]Average Cq values of four technical replicates

[b]DNA sample at 1 ul each target gene,

[c]singleplex vs multiplex. All comparisons are not significant.

Assay Specificity and Sensitivity. Gel electrophoresis and amplicon sequencing verified the amplification of target genes. *Tri5* and *actin* showed one band for singleplex reactions at less than 100 bp and between 100 and 200 bp, respectively (Fig. 1). Multiplex reactions show the same bands as singleplex reactions. Plasmids contained a 72 bp and 132 bp sequence for samples assigned to *Tri5* and *actin*, respectively. BLAST results returned *F. graminearum* for the 72 bp (95% identity, e-value: $2x10^{-18}$) and *Hordeum vulgare* or *Triticum aestivum actin* gene for the 132 bp (98% identity, e-value: $7x10^{-60}$). Taken together, these results confirm the specificity of the assay. The protocol detected 0.003 ng/ul (~3 pg/ul) of *F. graminearum* DNA and 0.03 ng/ul (~300 pg/ul) of barley DNA. Singleplex reactions showed one band for each dilution except 0.0003 ng/ul for *F. graminearum* (Fig. 2A). However, the three lowest concentrations of *barley* DNA (0.0003, 0.003, and 0.03 ng/ul) had the same band as the NTC. Multiplex reactions showed the same results, where the lowest concentration of *F. graminearum* DNA (0.0003

ng/ul) did not show any band, while the same three lowest concentrations of barley DNA displayed the same band as NTC (Fig. 2B). The *Cq* values ranged from 32 to 33 for these three barley DNA concentrations. These results determine the limit of detection at 0.003 ng/ul for *F. graminearum* DNA (Fig. 3A) and 0.3 ng/ul for *actin* (Fig. 3C). Standard curve analysis of the sensitivity tests in multiplex reactions showed a high correlation (R^2=0.999 for *Tri5* and 0.998 for *actin*) of the linear curve response with %E of 96.2 and 101.7 for *Tri5* (Fig. 3B) and *actin* (Fig. 3D), respectively (Fig. 3).

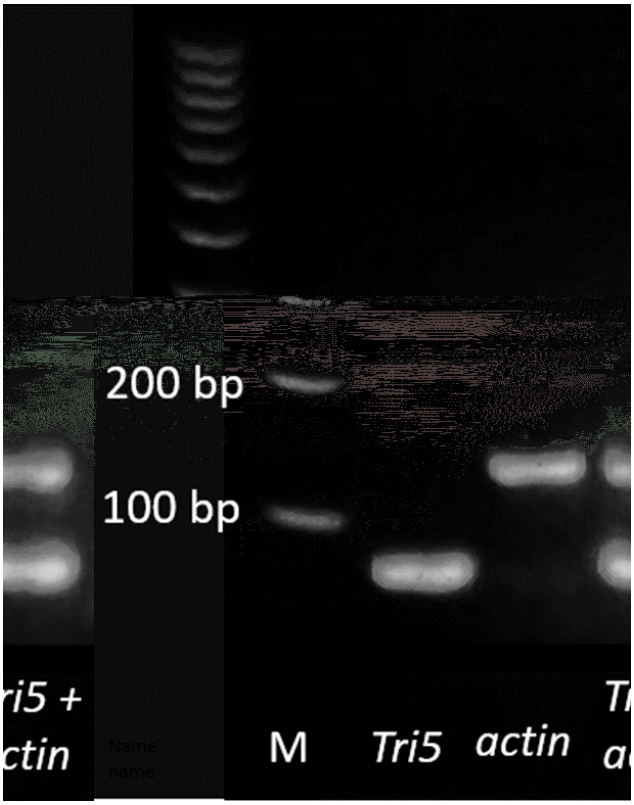


Figure 1. Specificity of *Tri5* and *actin* primers and probes in singleplex and multiplex reactions. *Tri5* is shown at 72 bp and *actin* at 132 bp.

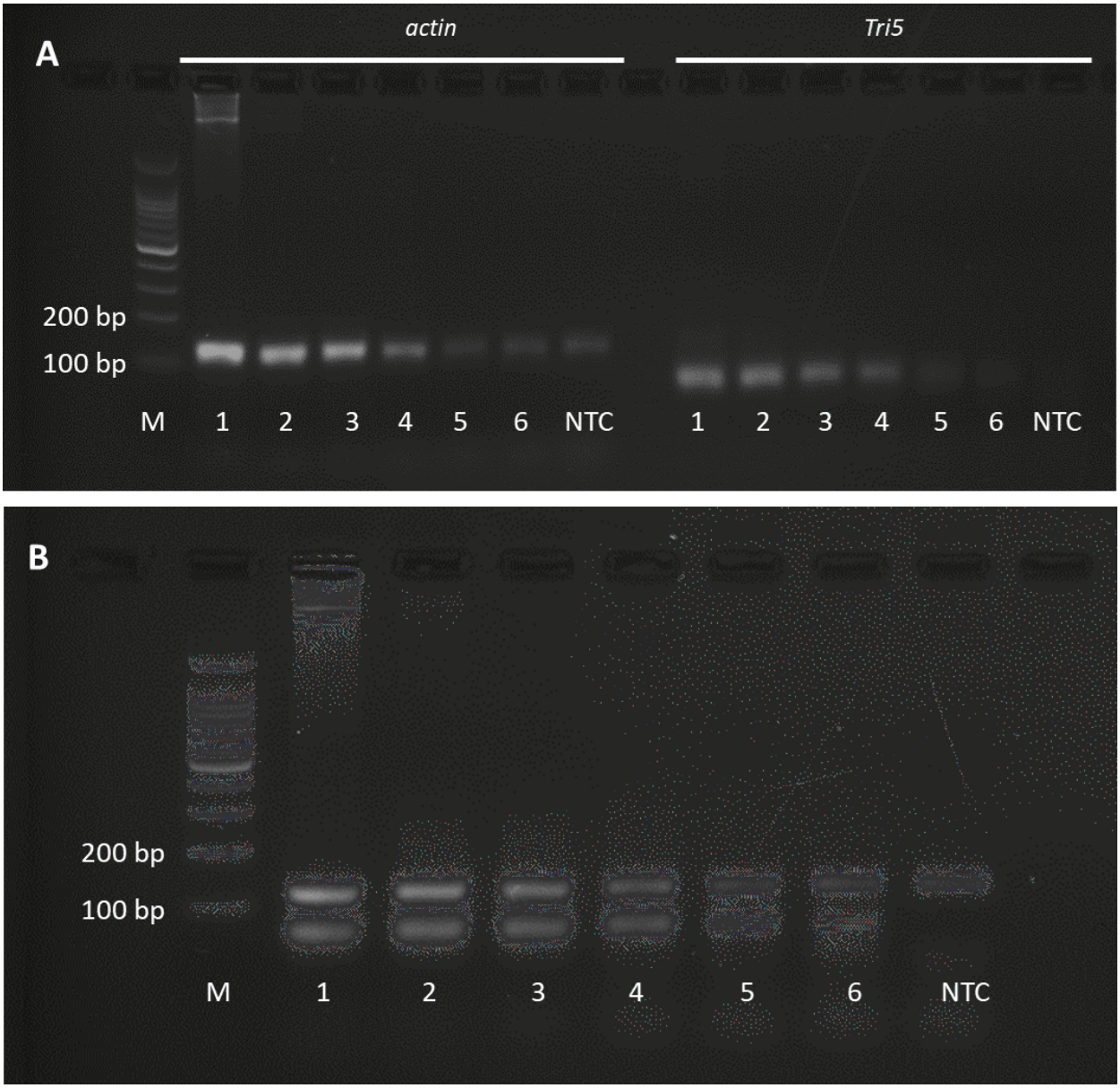
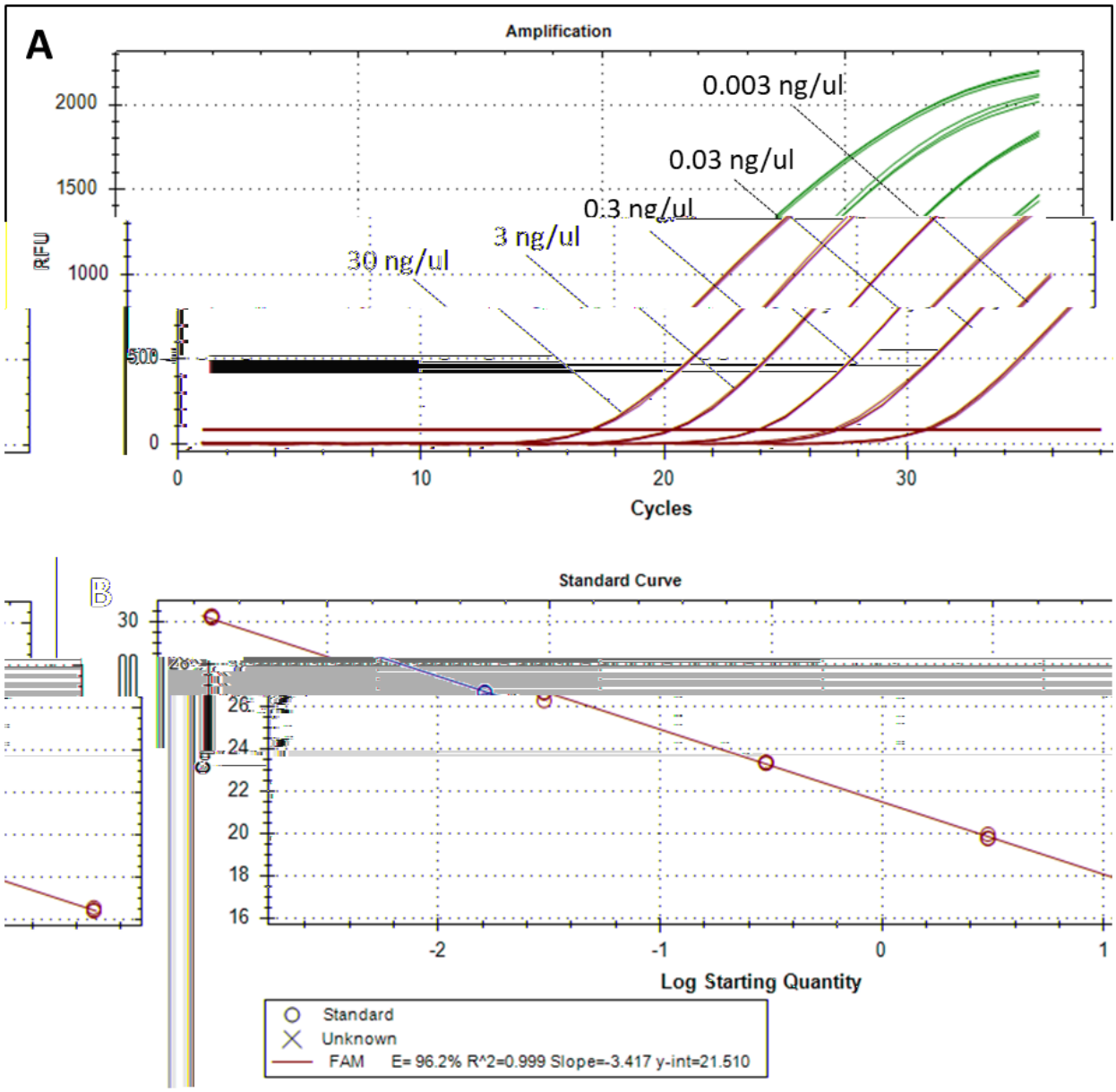


Figure 2. Sensitivity of *Tri5* and *actin* primers and probes in singleplex (A) and multiplex (B) reactions at varying concentrations showing the limit of detection at 0.003 ng/ul for *F. graminearum* DNA and 0.3 ng/ul for barley DNA. The *Tri5* gene is shown at 72 bp and the *actin* gene at 132 bp. M=marker, Lanes 1-6 are DNA concentrations (ng/ul): 30, 3, 0.3, 0.03, 0.003, 0.0003 for *Tri5* and 300, 30, 3, 0.3, 0.03, 0.003 for *actin*, NTC=no template control.


A
Amplification
0.003 ng/ul
0.03 ng/ul
0.3 ng/ul
3 ng/ul
30 ng/ul
RFU
2000
1500
1000
500
0
0
10
20
30
Cycles
B
Standard Curve
30
28
26
24
22
20
18
16
Cq
-2
-1
0
1
Log Starting Quantity
Standard
Unknown
FAM E= 96.2% R^2=0.999 Slope=-3.417 y-int=21.510

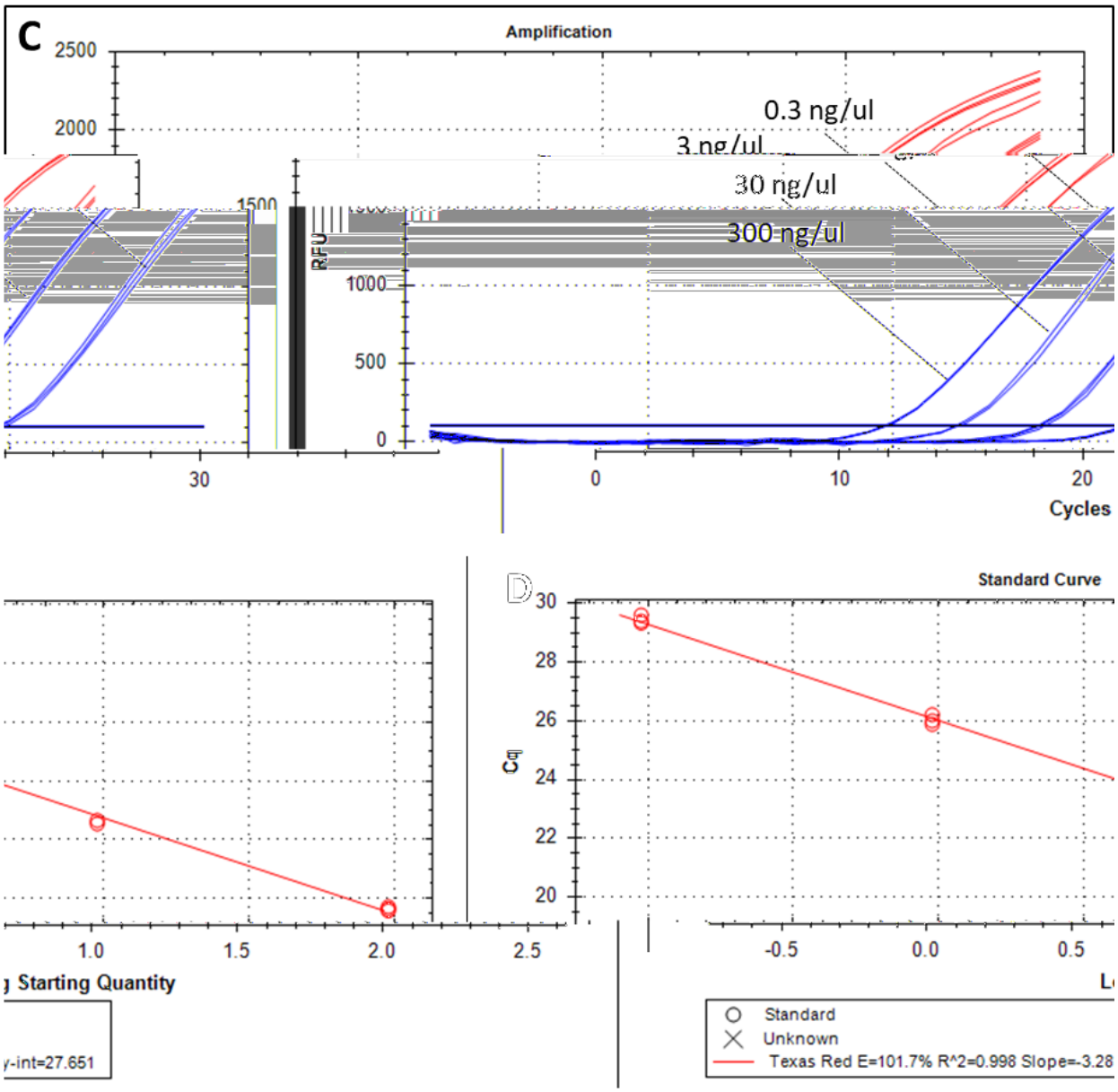


Fig 3. Standard real-time PCR sensitivity assay curve using serially diluted *F. graminearum* (A, B) and barley (C, D) DNA. The test was done in multiplex reactions but visualized separately due to overlapping amplification curves.

Assay Repeatability and Reproducibility. The repeatability test of nine replicates of all *F. graminearum* and barley DNA concentrations only showed slight variations in *Cq* values (Table 3). The reproducibility of the assay performed by different operators and on real-time PCR machines revealed insignificant differences in *Cq* values (Table 3). These results confirm the assay is repeatable and reproducible.

Table 3. Repeatability and reproducibility tests of multiplex real-time PCR assay of *Tri5* and *actin* genes at varying DNA concentrations.

Description	DNA Concentrations (ng/ul)								
	Tri5					*actin*			
	30	3	0.3	0.03	0.003	300	30	3	0.3
Repeatability Test									
Replicate 1	16.7	20.1	23.5	26.9	30.5	20.6	23.6	26.8	30.4
Replicate 2	16.7	20.1	23.8	26.8	30.6	20.9	23.5	26.9	30.2
Replicate 3	16.9	20.2	23.5	26.8	30.4	21.0	23.2	27.0	30.4
Replicate 4	16.5	20.3	23.6	26.9	30.8	21.2	23.3	26.9	30.5
Replicate 5	16.4	20.3	23.9	27.0	30.3	20.6	23.3	27.1	30.3
Replicate 6	15.9	20.6	23.7	26.8	30.5	21.2	23.6	27.1	30.4
Replicate 7	16.9	20.2	23.5	26.6	30.2	20.6	23.5	26.8	32.8
Replicate 8	16.7	20.4	23.6	26.7	30.3	21.0	23.5	26.8	30.3
Replicate 9	16.8	20.3	23.8	27.0	30.2	21.1	23.4	27.0	30.4
Mean	*16.6*	*20.3*	*23.6*	*26.8*	*30.5*	*20.9*	*23.4*	*26.9*	*30.6*
Coefficient of variation	*0.02*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.03*
Reproducibility Test[a]									
Equipment 1, Operator 1	16.3 ± 1.1	20.0 ± 0.9	23.3 ± 0.8	26.6 ± 1.2	30.0 ± 0.8	21.2 ± 1.2	24.4 ± 1.6	27.8 ± 1.5	30.7 ± 0.9
Equipment 1, Operator 2	16.7 ± 0.1	20.2 ± 0.1	23.4 ± 0.1	26.6 ± 0.0	30.3 ± 0.3	21.1 ± 0.0	24.0 ± 0.2	27.1 ± 0.1	30.8 ± 0.2
Equipment 2, Operator 2	16.4 ± 0.1	19.9 ± 0.1	23.5 ± 0.2	26.3 ± 0.2	30.1 ± 0.5	20.5 ± 0.3	23.2 ± 0.1	26.6 ± 0.3	30.2 ± 0.3
Mean	*16.5*	*20.0*	*23.4*	*26.5*	*30.1*	*21.0*	*23.9*	*27.2*	*30.6*
P-value	*0.88*	*0.93*	*0.94*	*0.94*	*0.90*	*0.80*	*0.66*	*0.68*	*0.73*

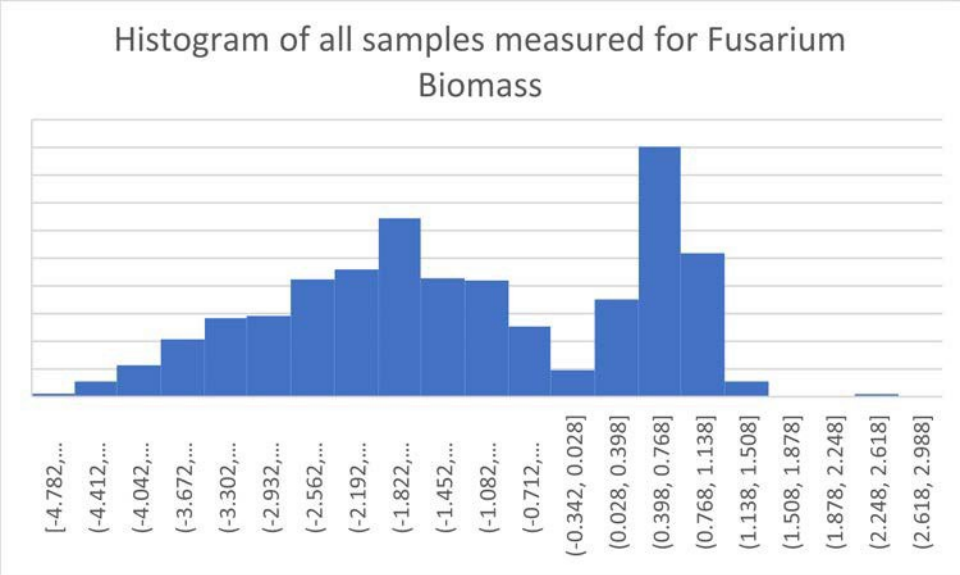
[a]Equipment 1 = Bio-rad CFX Opus 384, Equipment 2 = Bio-rad CFX 96 Touch

2. **Testing breeding material for *F. graminearum* biomass and saving DNA extractions as a possible repository for microbial fingerprinting.**

DNA was extract using Norgen biotech kits for over 4,000 samples from projects. The following table lists all of the projects that were sampled.

Projects	PI	Objective	Samples
2021 NABSEN	Baldwin	Obj. 1	1050
Training Population Kimberly (2020)	Klos	Obj. 1	500
Hulless Barley Diversity Panel	Smith	Obj. 1	1120
20 genotype - deep analysis	Geddes	Obj. 2	800
228 genotype - bulk analysis	Geddes	Obj. 2	912
	Total =		4382

An initial run of samples was measure for qPCR. Currently, the replications are being analyzed for accuracy. Results will be report back to the listed PIs to utilize in their breeding programs or scientific research.



b) What were the significant results?

We successfully developed and validated a TaqMan real-time PCR assay to address these issues and efficiently quantify mycotoxin-producing *F. graminearum* biomass. We based the assay on the *Tri5* gene of *F. graminearum* normalized by the barley *actin* gene. TaqMan multiplex PCR has the advantage of specific binding to target genes using probes and directly comparing the DNA of *F. graminearum* and barley in a single reaction. This reduces resources, time, and variability compared to running both target genes in separate reactions. Our protocol is distinctly developed and targeted to quantify *F. graminearum* biomass, expressed as the ratio of *Tri5* to the *actin* gene, and utilize it as a selection tool to aid breeding programs to consider both FHB infection and DON mycotoxin in breeding for FHB resistance.

Gel electrophoresis and amplicon sequencing confirmed the specificity of the protocol for both target genes. Sensitivity tests show a limit of detection of 0.003 ng/ul for *Tri5* and 0.3 ng/ul for *actin*. The protocol is sensitive to *Tri5*, allowing pathogen quantification at low concentrations. The *Tri5* has a lower quantification limit than actin, but this is not a concern as there is more *actin* than *Tri5* genes in DNA samples extracted from barley spikes or grains.

Developing resistant lines for FHB depends highly on DON levels, particularly in barley with lower tolerances for mycotoxin in malt production. This new high-throughput and reliable protocol is projected to have a faster turnaround time at minimum costs than conventional and commercial tests for DON. Availability of results without delay aids in making decisions for breeding for resistance, managing FHB, and supporting low-DON grain food products. Our future work includes determining the relationship of *F. graminearum* biomass to FHB severity and DON levels in different genotypes, thereby providing a greater understanding of the resistance of barley against *Fusarium* head blight.

c) List key outcomes or other achievements.

We supported four other projects in USWBSI and three PIs by testing their samples for Fusarium biomass. This information could be used to breed barley with increased resistance to FHB. Additionally, the publication of these results will allow other laboratories to efficiently test for Fusarium biomass in their experiments using our developed and validated primers.

3. What opportunities for training and professional development has the project provided?

One graduate student was trained on qPCR and was sent to USWBSI's annual meeting in 2022.

4. How have the results been disseminated to communities of interest?

A publication was recently submitted to Plant Health Program on the primers developed from this project. After further validation and data analysis the results from the qPCR will be shared with individual PIs for their projects.

A poster will be generated for the 2023 National FHB Forum on these results.

Publications, Conference Papers, and Presentations

Please include a listing of all your publications/presentations about your FHB work that were a result of funding from your FY21 grant award. Only citations for publications published (submitted or accepted) or presentations presented during the **award period** should be included.

Did you publish/submit or present anything during this award period?

x Yes, I've included the citation reference in listing(s) below.

☐ No, I have nothing to report.

Journal publications as a result of FY21 award

List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Include any peer-reviewed publication in the periodically published proceedings of a scientific society, a conference, or the like.

Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published [include DOI#]; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Navasca, A., Dangi S., Baldwin S. A., Jin Z., and Baldwin T., " Development and Validation of a Taq-man Multiplex qPCR Assay for High-Throughput Quantification of Fusarium graminearum Biomass in Barley Spikes and Grains" Plant Health Progress, Submitted. Acknowledged federal support: yes.

Books or other non-periodical, one-time publications as a result of FY21 award

Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series.

Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (book, thesis, or dissertation, other); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Other publications, conference papers and presentations as a result of FY21 award

Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication.