Barley disease screening: a multiplex digital droplet PCR approach for the detection of *Ramularia collo-cygni, Rhynchosporium graminicola* and *Pyrenophora teres*

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ABSTRACT

Barley is the most widely grown cereal crop in Ireland and faces substantial yield loss annually due to airborne, seed-borne, and soil-borne pathogens. The most prevalent and destructive of these include ramularia leaf spot, barley leaf scald and net blotch, caused by *Ramularia collo-cygni, Rhynchosporium graminicola* and *Pyrenophora teres* respectively. Infected seeds are considered as the major source of inoculum for these diseases. The absence of varietal resistance for these fungal pathogens suggests the need for better disease management strategies. Accurate pathogen detection is one of the most widely accepted disease management strategies. To facilitate such detections in complex samples, such as seed, we demonstrate two digital droplet PCR (ddPCR) assays, with the initial assay capable of detecting *R. graminicola*, *R.collo-cygni* and *P. teres*. For those identified as positive for *P. teres* samples, a form-specific assay was designed to distinguish between the two economically significant forms of *P. teres* ; *P. teres f. maculata* (Ptm; spot form of net blotch) and *P. teres f. teres* (Ptt; net form of net blotch). The assay offers sensitive and specific pathogen detection from barley seeds up to pico moles/µl level. Limits of detection for *R. graminicola, R. collo-cygni* and *P. teres* in the triplex assay were calculated as 1.67 cp/µl, 1.06 cp/µl and 4.21 cp/µl respectively. For form-specific assay, limits of detection were calculated as 1.44 cp/µl for Ptt and 0.82 cp/µl for Ptm assay. Finally, the efficacy of the assay was demonstrated by screening a collection of historical barley seeds.

1 | INTRODUCTION

After wheat and corn, barley is the 3rd most widely produced cereal crop in Europe, making up almost 20% of the total cereals produced (European Union Crop Production, 2024). Due to the combination of agronomic and climatic factors, the potential for high yields and favourable end-markets, barley accounts for approximately 70% of all cereals produced in Ireland (CSO, 2023). Unfortunately, as with all crops, barley can succumb to a spectrum of diseases that if left unchecked can seriously impact potential yields, both quantitatively and qualitatively. Most notable amongst these are the fungal foliar diseases ramularia leaf spot (RLS) caused by *Ramularia collo-cygni,* barley scald caused by *Rhynchosporium graminicola,* and the spot and net forms of net blotch (SFNB and NFNB), caused by *Pyrenophora teres f. maculata* (Ptm) and *P. teres f. teres* (Ptt), respectively (Huss et al. 1992;Avrova & Knogge, 2012; Liu et al., 2011).

As is the case with all plant diseases and pests, limiting initial inoculum/pest sources is critical to achieving sustainable control and is the principal tactic of integrated pest management (Barzman et al. 2015). For *R. collo-cygni*, *R. graminicola* and *P. teres* trash and stubble from the previous season's crops, volunteers and infected seed, all act as potential sources of initial inoculum. For *R. graminciola* and *P. teres,* it has been previously demonstrated that seed-borne infection represents a significant means of disease transmission and can directly impact disease levels and subsequent impacts on barley yields (Fountaine et al. 2010; Hysing & Wiik, 2013). For *R. collo-cygni*, whilst the pathogen can be readily detected in seed, and it has been demonstrated that it can grow from the seed into the developing seedling, the importance of seed-borne infection in the aetiology of RLS remains to be determined (Havis et al. 2014).

23 Traditionally seed has been tested for the presence of pathogens using culture dependent
24 techniques, whereby the test seeds are cultured on a selective growth media and subsequent
25 microbial growth is examined morphologically and/or microscopically to confirm the
26 contaminating species (Hiddink et al. 2023). However, there is a major disadvantage of
27 culture-dependent assays which is that they are restricted to those pathogens that can be
28 easily grown in culture and have a relatively fast growth rate. Of the three major barley
29 pathogens identified above, it is only possible to routinely identify *P. teres* through culture-
30 dependent assays as both *R. graminicola* and *R. collo-cygni* are relatively slow growing and
31 are outcompeted by other fungi (pathogenic or non-pathogenic) present in barley seed.
32 Identification of *P. teres* in culture can also be difficult as it can be mistaken for *P. graminea*,
33 which exhibits a similar morphology in culture (Liu et al. 2011). Similarly, it is not possible to
34 distinguish between Ptm and Ptt when grown in culture or by using osmotic or blotter tests
35 (Brodal, 1997). More recently developed detection methods that rely on detection of nucleic
36 acids (either DNA or RNA) have improved specificity compared to culture-based detection
37 methods. They also allow a wider range of pathogens to be detected, whilst simultaneously
38 reducing costs and run time (Elnifro Elfath et al. 2000).

39 Seed samples are unfortunately often complex matrixes, which can inhibit or impair DNA
40 based reactions and assay sensitivities (Lu et al. 2020), in particular those such as quantitative
41 polymerase chain reaction (qPCR) on which quantification is based on comparable efficiencies
42 of reactions required between the test sample and samples/standards of a known DNA
43 concentration (Hindson et al. 2013). Digital droplet PCR (ddPCR, also referred to as digital PCR
44 (dPCR) or crystal digital PCR (cdPCR) depending on the platform used) is a third generation of
45 PCR in which the starting DNA sample is partitioned through the use of microfluidics into
46 thousands of samples which subsequently undergo PCR reactions. By coupling partitioning

and PCR with fluorescent dye based DNA probes it is possible to use endpoint analysis to confirm the presence or absence of the targeted DNA in partitioned samples (Mao et al. 2019). Through the use of Poisson's Law, which estimates the proportion of droplets containing the target DNA, it is then possible to determine the total copies of the target DNA in the starting sample (Quan et al. 2018). As quantification is based on an end point reaction ddPCR is not sensitive to PCR inhibitors while qPCR is strongly influenced by the efficiencies of the reaction (Basu, 2017). Therefore, ddPCR allows for the accurate and sensitive quantification of specific DNA targets, even in complex matrices, making it an ideal platform for the detection of pathogens in cereal seeds.

Different PCR based methods have been developed for each of the commonly occurring barley pathogens, with qPCR assays available for both *P. teres* and *R. graminicola* (Leisova et al. 2006); Fountaine et al. 2007), and a dPCR based method for detection of *R. collo-cygni* recently published by Knight et al. (2022). To exploit the multi-spectral capacity of the Stilla 3-colour Nacia® ddPCR system, which allows simultaneous detection of the fluorescence emitted from three spectrally different fluorophores, here we combine the *R. graminicola* and *R. collo-cygni* assays of Fountaine et al. (2007) and Knight et al. (2022), with a *P. teres* probe based assay to provide a triplex ddPCR assay to simultaneously detect and quantify the presence of the three pathogens from historical barley seed samples. For the samples positive for *P. teres*, a further form-specific ddPCR assay was designed to detect and differentiate between the spot and net form of *P. teres.*

2 | MATERIALS AND METHODS

A multistage process of assay design, optimisation and application was developed and is outlined in Figure 1.

70 **2.1 | Fungal strains and DNA extraction**

71 All fungal cultures used in the study (Supplementary Table 1) were acquired from the
72 phytopathological collection in Teagasc, Oak Park, Co. Carlow. DNA was extracted from these
73 cultures following freeze drying (for 24 hours) and homogenisation (with Retsch Mixer Mill
74 MM 400 at a frequency of 20 Hz for 5 minutes) using a Qiagen DNeasy Plant Mini kit (Qiagen,
75 Germany), following the manufacturer's instructions. Extracted DNA was quantified using
76 Invitrogen™ Qubit™ Fluorometer with a Qubit™ 1X dsDNA BR Assay (Invitrogen, US). For
77 some of the cultures (See Supplementary Table 1: Isolates utilized in the study), only DNA was
78 provided and the depositor confirmed presence of fungal DNA with PCR.

79 **2.2 | Assay designs and initial optimization**

80 For the initial ddPCR triplex assay to detect and distinguish between the three target fungal
81 pathogens, the published fluorescence probe based assays for the detection of *R. collo-cygni*
82 (Knight et al. 2022) and *R. secalis* (Fountaine et al. 2007) were selected. The *R. secalis* probe
83 length was one base pair shorter than the original probe described by Fountaine et al. (2007).
84 A separate set of primers and probe to detect *P. teres* was designed using Primer 3Plus
85 (https://www.primer3plus.com/index.html) based on the Internal Transcribed Spacer (ITS)
86 region of *P. teres* (OM471876.1). To further distinguish between Ptm and Ptt primers were
87 deployed from Poudel et al. (2017) while probes were designed using Ptm and Ptt mRNA
88 sequences from the same (accession numbers Ptm: KX909562.1 and Ptt: KX909556.1).
89 However, for Ptm detection, a new reverse primer was designed using the same sequence in
90 order to reduce the amplicon length for optimal qPCR efficiency (Van Holm et al. 2021) Online
91 tool; PrimerQuest™ (Integrated DNA Technologies) was deployed for primer and probe
92 designing. tripl Primers and probes were ordered from Integrated DNA Technologies

93 (Europe). Details of all primers and probes are summarized in Table 1. Initial primer and probe
94 optimisation, including identifying annealing temperatures, was conducted using routine PCR
95 and visualised using agarose gel electrophoresis.

96 **2.3 | ddPCR optimisation**

97 Following these initial validation steps, the assays were transferred to ddPCR system (naica®
98 Crystal Digital PCR 3 colour system, Stilla Technologies, France). ddPCR using the naica®
99 Crystal Digital PCR 3 colour is separated into four stages: 1) the reaction mixture (primers,
100 probes, DNA of the pathogen and host and ultrapure water) is partitioned into nano-sized
101 droplets on a chip (up to 17k on a Ruby chip or 30k on a Sapphire chip), 2) partitioned samples
102 undergo up to 40 PCR cycles, 3) once the PCR is complete a scan of the chip is taken under
103 different wavelengths (corresponding to the excitation of the FAM, HEX and Cy5 dyes) is
104 undertaken, and 4) scanned chips are analysed to identify droplets with the target DNA
105 amplified based on presence or absence of the specified molecular probe-associated
106 fluorescence. In the present study all reactions were conducted using the Ruby Chip (up-to
107 17k droplets per chamber) and 5X naica® multiplex PCR MIX (Stilla Technologies, France) and
108 only reactions with >10k analysable droplets were included.
109 An initial series of reactions were performed to optimize concentrations of primers and
110 probes required to differentiate between positive and negative droplets. Reaction mixtures
111 were prepared in accordance with the guidelines provided by Stilla Technologies, consisting
112 of 1 µl of Buffer A, 0.2 µl of Buffer B (both Buffer A and Buffer B were supplied as part of the
113 5X naica® multiplex PCR MIX), 0.25 µl each of the primers (0.05 µM final concentration), 0.15
114 µl of each probe (0.06 µM final concentration), 0.85 µl of barley DNA (17 ng/µl), 1 µl of original
115 pathogen DNA was used as a template (0.1 ng/µl) and nuclease free water (Fisher Scientific,

116 UK) was added to reach a final volume of 5 µl. All PCR reactions were performed in triplicates
117 and in the presence of pathogen-free barley DNA as a matrix; extracted from two-week barley
118 seedlings (variety: RGT Planet, Goldcrop Ltd, Ireland) grown under controlled conditions
119 (16 h of light, 8 h of dark, at 20 and 16 °C). Thermal cycling conditions for both assays are as
120 follows; partition at 25°C followed by 95°C for 3 minutes, then 5 cycles of 95°C for 10 seconds
121 followed by 66°C for 30 seconds, and then 35 cycles at 95°C for 10 second and 64°C for 15
122 second. A compensation matrix was designed according to the guidelines provided by Stilla
123 Technologies, to normalize the fluorescence overlap and spill over.

124 **2.4 | Determining Limits of Blank (LoB) and Limits of Detection (LoD)**

125 Limit of blank (LoB) and Limit of detection (LoD) for the assays were calculated in multiplex
126 fashion and in accordance with the guidelines provided by Stilla Technologies. LoB can be
127 defined as the maximum target concentration expected in the blank sample with a probability
128 PLoB = 1 – α (typically 1 – α = 95% where α = 5% of false positives), where α is the probability
129 of making a false positive decision in a real blank sample (no DNA of any target except the
130 background/barley DNA). Whereas, LoD is defined as the minimum concentration, a non-
131 zero, statistically significant value, that can be detected with a probability of 1 – β (typically 1
132 – β = 95% where β = 5% of false negatives), β is the probability making a false negative decision
133 in a real positive sample.

134 To determine the LoB, 30 blank (negative for the target for which LoB is being performed)
135 reaction replications were performed for each target. To calculate LoD, ten-fold serial
136 dilutions of a known target DNA concentration was performed and a non-zero, statistically
137 significant concentration detected by the assay, designated as low-level sample, was
138 calculated. Afterwards, five independent low-level samples were prepared and six reaction
139 replications of each were performed. Based on the copy per microliter (cp/µl) measured by

140 the fluorescence detector, LoD and LoB was calculated by using the online tool provided by
141 Stilla Technologies (https://www.stillatechnologies.com/digital-pcr/statistical-tools/limit-
142 detection/).

143 **2.5 | Linear dynamic range of the assay**

144 To calculate the relationship between the expected and measured quantities of each of the
145 target pathogen DNA, linear dynamic range of the assay was calculated. To achieve this, a 10-
146 fold dilution series (0.01–0.00001 ng/µl) of each pathogen DNA (*R. collo-cygni*, *R. graminicola*
147 and *P. teres*) was prepared and tested individually whilst keeping a constant DNA
148 concentration of the other two pathogens (0.1 ng/µl) in the reaction mixture. The log10
149 values of concentration against copy number/µl (cp/ µl) were plotted and correlation was
150 calculated in Microsoft Excel.

151 **2.6| Specificity of the assays**

152 Specificity of triplex and form-specific assays was performed by challenging the system with
153 a number of barley pathogens/barley microflora including *Botrytis cinerea (Bc),*
154 *Parastagonospora nodorum (Pn), Alternaria alternate (Aa), Fusarium graminearum (Fg),*
155 *Microdochium nivale (Mn)* and *Fusarium culmorum (Fc)* accessions that are stored as part of
156 the Phytopathological collection in Teagasc, Oak Park, Co. Carlow. Additionally, specificity of
157 the form-specific was also verified against *R. collo-cygni* and *R. graminicola* in a singleplex
158 reaction. Each of the pathogen DNA was tested in an individual ddPCR reaction.

159 **2.7 | Detection of pathogens in seed and leaf samples**

160 To validate the reliability of the assays (triplex and form-specific assay) for naturally infected
161 barley samples, historical winter (35 samples) and spring (32 samples) barley seed DNA from
162 2016–2018, with variable susceptibility to leaf blotches, were evaluated using the assays. The
163 details of seed DNA extraction is described in Mulhare et al. (2021). Any droplet population

above LoD of the respective assay was considered positive. Samples positive for *P. teres* were further tested by the form specific assay to confirm *P. teres* form.

Volunteers from the previous season crop are one of the sources of inoculum for RLS, barley leaf scald and net blotch. Efficacy of the triplex assay was also assessed in wheat volunteers from barley field with visual symptoms of RLS (Teagasc wheat trials in Knockbeg, Ireland (2023)). Leaves were freeze dried and DNA was extracted as described above. Extracted DNA (1 µl) was used as a template in the reaction mixture described in section 2.3.

3 | Results

3.1 | Assay design and optimization

For the detection of *R. collo-cygni* and *R. graminicola*, primers and probes were deployed from published assays targeting translation elongation factor 1-alpha gene and Cytochrome b genes respectively (Fountaine et al. 2007; Knight et al. 2022). However, for *P. teres* detection and to distinguish between both Ptt and Ptm it was necessary to design novel fluorescent probes (Table 1). The initial triplex assay was specific for the three pathogens, with no fluorescence overlap observed between the different probes-pathogen combinations (Figure 2A). In triplex assay, we observed 5044 cp/µl and 10210 cp/µl for *R. graminicola* and *P. teres,* for *R. collo-cygni and P. teres* 156.4 and 10993 cp/µl and for *R. graminicola, R. collo-cygni* sample 5600 and 164.4 cp/µl respectively. *teres* form specific assay there was no fluorescence overlap between the probe-pathogen combination, demonstrating specificity of the assay. We observed 112.8 cp/µl in FAM channel and 7086 cp/µl in Cy5 channel in Ptt sample, 576.6 cp/µl in HEX and 20267 cp/µl in Cy5 channel in Ptm sample, for both forms we observed 186.5 cp/µl in FAM, 501.3 cp/µl in HEX and 26113 cp/µl in Cy5 channel (Figure 2B). For *R. graminicola, R. collo-cygni and P. teres* the LoB were calculated to be 0.68, 0, and 1.34 cp/µl

187 respectively. For the *P. teres* form-specific assay and LoB of 0 cp/µl was calculated for both
188 Ptt and Ptm. Limits of detection (LoD) for *R. graminicola, R. collo-cygni* and *P. teres* in the
189 triplex assay were calculated as 1.67 cp/µl, 1.06 cp/µl and 4.21 cp/µl respectively.
190 Additionally, LoD was calculated as 1.44 cp/µl for Ptt assay and 0.82 cp/µl for Ptm assay. No
191 positive droplets, irrespective of probe, were detected in negative control (barley DNA only)
192 (Figure 2A & B).

193 **3.2 | Linear dynamic range of the assay**

194 To determine the accuracy of the assay, the linear dynamic range of the assay was ascertained
195 by correlating the projected DNA copies of each target in a sample and copy numbers
196 detected. For each probe-pathogen combination R^2 values of >0.99 were calculated. Linear
197 dynamic range of the assay also indicated the lower limit of detection of the assay, which is
198 0.00001 ng/µl (Figure 3). The linear dynamic range of the *P. teres* form specific assay was not
199 determined as it was specifically designed to distinguish between forms following initial
200 detection of *P. teres* using the triplex assay. Details of cp/µl for linear range is provided in
201 Supplementary information (Linear dynamic range.xlsx).

202 **3.3 | Specificity of the assay**

203 Considering the potential application of our assays, for screening seeds and leaf samples from
204 the field, specificity of both the assays was investigated against a range of fungi that may be
205 found on or in barley seeds and leaves. No positive droplets were detected in any of the
206 fluorescent channels for any of the fungi tested, indicating that the assays are highly specific
207 (Figure 4A & B).

208 **3.4 | Detection of barley pathogens in wheat**

209 Wheat leaf DNA was also tested for the presence of *R. collo-cygni.* The assay showed a 469.7
210 cp/µl in sample 9, 4546 cp/µl and 1557 cp/µl in sample 11 and 13 respectively of *R. collo-cygni*

DNA from symptomatic wheat leaves. However, wheat samples displayed positive droplets for the presence of *R. graminicola and P. teres* DNA in the samples (Figure 5). In order to rule out the *P. tritici-repentis* cross reactivity with the triplex assay, we performed the form-specific assay and we observed, 0.86 to 1.18 cp/µl of Ptt across two samples (Supplementary Figure 2).

3.5 | Detection of *R. collo-cygni*, *R. graminicola* and *P. teres* in barley seed samples

To determine the application of assays to test the presence of pathogens in naturally infected barley DNA samples, previously extracted from seeds of winter and spring barley from 2016 (Kildea et al. 2024) were tested. Only samples exhibiting cp/µl equal to or above the limits of detection, as identified above, were deemed positive for the respective pathogens. Of the 35 winter barley samples tested, 16 samples were positive for *R. collo-cygni*, two samples were positive for *R. graminicola,* while 19 were positive for *P. teres.* For the 32 spring barley samples, 15 were positive for *R. graminicola*, 25 samples were positive for *R. collo-cygni,* and 25 positive for *P. teres*. Nine of the spring and winter barley samples that were found to be positive for *P. teres* were tested to determine the form determination. None of the samples tested positive for the presence of *P. teres f. maculata*. In the case of winter barley, eight out of nine samples were positive for *P. teres f. maculata* and two samples were positive for *P. teres f. teres* as well as *P. teres f. maculata* (Supporting information seed testing data).

4 | Discussion

Clean seeds are undeniably important for healthy crops and this is reliant on early detection of specific disease agents, which can further facilitate the execution of IPM based disease control strategies (Dussart et al. 2020; Tsedaley, 2015). The ddPCR assays described offer a specific, reliable and sensitive means of detecting and quantifying three of the most economically important barley pathogens in a single reaction. The end point nature of the

235 digital PCR assay makes it ideal for detecting the different pathogens in complex plant
236 materials, such as seeds (Liu et al. 2011).

237 To validate the efficacy of our assays and investigate the prevalence of *R. collo-cygni, R.*
238 *graminicola* and *P. teres* in field samples, we screened a selection of historical barley seed
239 samples collected from 2016 to 2018 and wheat leaves sampled from volunteer wheat plants
240 in a winter barley field in 2023. Unsurprisingly, the barley seed samples were contaminated
241 with variable levels of the three pathogens, with a proportion coinfected with all three
242 pathogens. All three pathogens were detected in the winter wheat leaves, the latter result is
243 unsurprising given the fact that the leaves tested were collected from wheat volunteers
244 collected from a winter barley field. Whilst *R. collo-cygni* has previously been reported in
245 wheat in Ireland (Zia et al. unpublished), the detection of *R. secalis* and *P. teres* in the sample
246 may simply reflect the presence of both pathogens on the wheat leaf surface rather than
247 colonisation of the leaf tissue. This highlights a potential limitation of the assays. As they are
248 based on the detection of DNA of the respective pathogens, they cannot determine if the
249 detected pathogen was actively colonising the plant tissue, or if the pathogen detected was
250 even viable. However, it is important to note that these limitations are not restricted to the
251 assays developed and described in this paper but are a drawback of all DNA-based detection
252 methods (Ficetola et al. 2015). It is therefore critical that any positive detections are assessed
253 in the context of detection (host) and the potential risk(s) posed by the pathogen. As
254 mentioned previously it is more likely that the positive detections for *R. graminciola* and *P.*
255 *teres* from the wheat volunteers are consequential to levels of both diseases in the
256 surrounding barley crop. However, it is worth highlighting that in addition to *R. collo-cygni*
257 being noted as able to colonise wheat, Ptt has been reported as causing disease on wheat in
258 Hungary (Tóth et al. 2008), Russia (Mikhailova et al. 2010) and most recently in Brazil (Garozi

259 et al. 2020). Further analysis of wheat grains and/or a wider wheat disease survey is
260 warranted to determine if either pathogen is more widespread in wheat crops.

261 Those barley seed samples that presented positive for *P. teres* were subsequently tested to
262 determine what form of *P. teres* was present. Amongst the spring barley samples tested only
263 Ptt was present. For the winter barley seed samples both Ptt and Ptm were detected, with a
264 number of samples positive for both. All samples with detectable levels of Ptm, irrespective
265 of the presence of Ptt, were from the cultivar KWS Tower, which had by the late 2010s
266 become highly susceptible to *P. teres*, and in particular Ptm (Recommendation list in
267 supplementary information and author's unpublished data). Positive detection of both Ptt
268 and Ptm could indicate of a potential Ptt/Ptm hybrid, however based upon the number of
269 droplets positive for both in the test samples this appears unlikely. The presence of markers
270 for both forms does suggest that hybrids may arise, with recent reductions in fungicide
271 sensitivity and efficacy in Western Australia associated with such a hybrid (Mair et al. 2020;
272 Turo et al. 2021).

273 It is worth noting that within the multi-pathogen assay the region targeted for *P. teres* was
274 ITS region, whilst in the form specific assay we targeted the Ptt form-specific region PttQ5
275 mRNA sequence and the Ptm form-specific region PtmQ11 mRNA sequence. The copy
276 number for ITS in different fungi can vary considerably (Lofgren et al. 2019) and the copy
277 number of PtmQ11 and PttQ5 are unknown in *P. teres.* As a number of samples that tested
278 positive for *P. teres* in the multi-pathogen assay, were neither positive for either Ptt or Ptm
279 suggests PtmQ11 and PttQ5 have a lower copy number compared to ITS region in *P. teres.*
280 Therefore, ~ 15 cp/µl of *P. teres* sequence are required to confirm the presence of Ptt or Ptm
281 from the samples, as observed in the case of historical seed samples. Moreover, high droplet

population in *P. teres* ITS could potentially be the consequence of similarity between *P.teres* and *P. graminea* ITS regions (Supplementary Figure 1), therefore, we suggest further exploration of the historical seed samples.

As infected seeds are a likely source for the introduction of new pathogens and resistance /virulence mechanisms across the globe, including the three pathogens discussed, early detection and subsequent management in seed stocks will help minimise their spread (Stam et al. 2019). Therefore, molecular seed screening for pathogen levels and identification of the presence of fungicide resistance or virulence alleles should be a regular practice prior to seed transportation. This ddPCR assay can be an efficient tool for regular seed screening. Furthermore, establishment of pathogen threshold levels by deploying ddPCR, coupled with the rejection of seeds with high pathogen levels, can minimize the risk of future outbreaks. Seed is only one of the sources of these diseases, however prevention is the key to suppression.

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Conflict of interest declaration

None declared.

Data availability statement

The data that support the findings of this study are available on reasonable request from the corresponding author.

ReferencesAvrova, A., & Knogge, W. (2012) *Rhynchosporium commune*: a persistent threat to barley cultivation. *Molecular Plant Pathology*, 13(9), 986-997. https://doi.org/https://doi.org/10.1111/j.1364-3703.2012.00811.x

Barzman, M., Bàrberi, P., Birch, A. N. E., Boonekamp, P., Dachbrodt-Saaydeh, S., Graf, B., Hommel, B., Jensen, J. E., Kiss, J., Kudsk, P., Lamichhane, J. R., Messéan, A., Moonen, A.-C., Ratnadass, A., Ricci, P., Sarah, J.-L., & Sattin, M. (2015) Eight principles of integrated pest management. *Agronomy for Sustainable Development, 35*(4), 1199-1215. https://doi.org/10.1007/s13593-015-0327-9

Basu, A. S. (2017) Digital assays part I: Partitioning statistics and digital PCR. *SLAS Technology, 22*(4), 369-386. https://doi.org/https://doi.org/10.1177/2472630317705680

Bates, J. A., Taylor, E. J., Kenyon, D. M., & Thomas, J. E. (2001) The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. *Molecular Plant Pathology, 2*(1), 49-57. https://doi.org/10.1046/j.1364-3703.2001.00049.x

Bingham, I. J., Blake, J., Foulkes, M. J., & Spink, J. (2007) Is barley yield in the UK sink limited?: II. Factors affecting potential grain size. *Field Crops Research, 101*(2), 212-220. https://doi.org/https://doi.org/10.1016/j.fcr.2006.11.004

Brodal, G. (1997) Comparative Tests with the osmotic blotter method for detection of *Drechslera* spp. in barley seeds. In: Seed Health Testing – Progress Towards the 21st Century. (Eds.) *J. D. Hutchins & J. C. Reeves*, pp. 211–218.

Dussart, F., Creissen, H. E., & Havis, N. D. (2020) *Ramularia collo-cygni*-an enemy in waiting. In: *eLS* (pp. 1-8). Advance online publication. https://doi.org/10.1002/9780470015902.a0028896

Elnifro Elfath, M., Ashshi Ahmed, M., Cooper Robert, J., & Klapper Paul, E. (2000) Multiplex PCR: optimization and application in diagnostic virology. *Clinical Microbiology Reviews, 13*(4), 559-570. https://doi.org/10.1128/cmr.13.4.559

European Union Crop Production. (2024) https://ipad.fas.usda.gov/countrysummary/default.aspx?id=E4.

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., & Pompanon, F. (2015) Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources, 15*(3), 543-556.

Fountaine, J. M., Shaw, M. W., Napier, B., Ward, E., & Fraaije, B. A. (2007) Application of real-time and multiplex Polymerase Chain Reaction assays to study leaf blotch epidemics in barley. *Phytopathology®, 97*(3), 297-303. https://doi.org/10.1094/phyto-97-3-0297

Fountaine, J. M., Shaw, M. W., Ward, E., & Fraaije, B. A. (2010) The role of seeds and airborne inoculum in the initiation of leaf blotch (*Rhynchosporium secalis*) epidemics in winter barley.

Plant Pathology, 59(2), 330-337. https://doi.org/https://doi.org/10.1111/j.1365-3059.2009.02213.x

Garozi, R., Angelotti, P., Feksa, H. R., & Tessmann, D. J. (2020) First report of *Pyrenophora teres f. teres* causing leaf spot on wheat in Brazil. *Plant Disease, 104*(12), 3254-3254. https://doi.org/10.1094/pdis-03-20-0570-pdn

Havis, N. D., Nyman, M., & Oxley, S. J. P. (2014) Evidence for seed transmission and symptomless growth of *Ramularia collo-cygni* in barley (*Hordeum vulgare*). *Plant Pathology*, 63(4), 929-936. https://doi.org/https://doi.org/10.1111/ppa.12162

Hiddink, G. A., Willmann, R., Woudenberg, J. H. C., & Souza-Richards, R. (2023) Seed health testing: doing things right. *PhytoFrontiers™, 3*(1), 71-74. https://doi.org/10.1094/phytofr-03-22-0029-fi

Hindson, C. M., Chevillet, J. R., Briggs, H. A., Gallichotte, E. N., Ruf, I. K., Hindson, B. J., Vessella, R. L., & Tewari, M. (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nature Methods, 10*(10), 1003-1005. https://doi.org/10.1038/nmeth.2633

Huss, H., Mayrhofer, H., & Iugolič, E. (1992) *Ramularia collo-cygni* Sutton & Waller fungi imperfecti, ein wirtschaftlich bedeutender parasit der gerste in der Steiermark. *Mitteilungen des Naturwissenschaftlichen Vereines für Steiermark*, 122, 87-95.

Hysing, S.-C., & Wiik, L. (2013) The role of seed infection level and fungicide seed treatments in control of net blotch in barley. *European Journal of Plant Pathology, 137*(1), 169-180. https://doi.org/10.1007/s10658-013-0230-7

Kildea, S., Mulhare, J., Zia, R., Hutton, F., & Creissen, H. (2024) Presence and prevalence of *Ramularia collo-cygni* SDHI resistance in Irish barley seed. *Journal of Plant Diseases and Protection, 131*(4), 1233-1238. https://doi.org/10.1007/s41348-024-00910-4

Knight, N. L., Moslemi, A., Begum, F., Dodhia, K. N., Covarelli, L., Hills, A. L., & Lopez-Ruiz, F. J. (2022) Detection of *Ramularia collo-cygni* from barley in Australia using triplex quantitative and droplet digital PCR. *Pest Management Science, 78*(4), 1367-1376. https://doi.org/https://doi.org/10.1002/ps.6753

Leisova, L., Minarikova, V., Kucera, L., & Ovesna, J. (2006) Quantification of *Pyrenophora teres* in infected barley leaves using real-time PCR. *Journal of Microbiological Methods,* 67(3), 446-455. https://doi.org/https://doi.org/10.1016/j.mimet.2006.04.018

Liu, Z., Ellwood, S. R., Oliver, R. P., & Friesen, T. L. (2011) *Pyrenophora teres*: profile of an increasingly damaging barley pathogen. *Molecular Plant Pathology, 12*(1), 1-19.

Lofgren, L. A., Uehling, J. K., Branco, S., Bruns, T. D., Martin, F., & Kennedy, P. G. (2019) Genome-based estimates of fungal rDNA copy number variation across phylogenetic scales and ecological lifestyles. *Molecular Ecology, 28*(4), 721-730.

Lu, Y., Zhang, H.-j., Zhao, Z.-j., Wen, C.-l., Wu, P., Song, S.-h., Yu, S.-c., Luo, L.-x., & Xu, X.-l. (2020) Application of droplet digital PCR in detection of seed-transmitted pathogen *Acidovorax citrulli*. *Journal of Integrative Agriculture, 19*(2), 561-569. https://doi.org/https://doi.org/10.1016/S2095-3119(19)62673-0

Mair, W. J., Thomas, G. J., Dodhia, K., Hills, A. L., Jayasena, K. W., Ellwood, S. R., Oliver, R. P., & Lopez-Ruiz, F. J. (2020) Parallel evolution of multiple mechanisms for demethylase inhibitor fungicide resistance in the barley pathogen *Pyrenophora teres f. sp. maculata*. *Fungal Genetics and Biology, 145*, 103475. https://doi.org/https://doi.org/10.1016/j.fgb.2020.103475

Mao, X., Liu, C., Tong, H., Chen, Y., & Liu, K. (2019) Principles of digital PCR and its applications in current obstetrical and gynecological diseases. *American Journal of Translational Research*, 11(12), 7209-7222

Mikhailova, L. A., Ternyuk, I. G., & Mironenko, N. V. (2010) *Pyrenophora teres*, an agent causing wheat leaf spot. *Microbiology, 79*(4), 561-565. https://doi.org/10.1134/S0026261710040223

Mulhare, J., Creissen, H. E., & Kildea, S. (2021) Effectiveness of varietal resistance and risk prediction for the control of ramularia leaf spot of barley under Irish growing conditions. *Crop Protection, 139*, 105317. https://doi.org/https://doi.org/10.1016/j.cropro.2020.105317

Poudel, B., Ellwood, S. R., Testa, A. C., McLean, M., Sutherland, M. W., & Martin, A. (2017) Rare *Pyrenophora teres* hybridization events revealed by development of sequence-Specific PCR markers. *Phytopathology, 107*(7), 878-884. https://doi.org/10.1094/phyto-11-16-0396-r

Quan, P. L., Sauzade, M., & Brouzes, E. (2018) dPCR: A Technology Review. *Sensors* (Basel), 18(4). https://doi.org/10.3390/s18041271

Stam, R., Sghyer, H., Tellier, A., Hess, M., & Hückelhoven, R. (2019) The current epidemic of the barley pathogen *Ramularia collo-cygni* derives from a population expansion and shows global admixture. *Phytopathology®, 109*(12), 2161-2168. https://doi.org/10.1094/phyto-04-19-0117-r

Tóth, B., Csősz, M., Kopahnke, D., & Varga, J. (2008) First report on *Pyrenophora teres* causing lesions of wheat leaves in Hungary. *Plant Pathology, 57*(2), 385-385. https://doi.org/https://doi.org/10.1111/j.1365-3059.2007.01746.x

Tsedaley, B. (2015) Review on seed health tests and detection methods of seedborne diseases. *Journal of Biology, Agriculture and Healthcare, 5*(5).

Turo, C., Mair, W., Martin, A., Ellwood, S., Oliver, R., & Lopez-Ruiz, F. (2021) Species hybridisation and clonal expansion as a new fungicide resistance evolutionary mechanism *in Pyrenophora teres* spp. *bioRxiv*, 2021.2007. 2030.454422.

436 Van Holm, W., Ghesquière, J., Boon, N., Verspecht, T., Bernaerts, K., Zayed, N., Chatzigiannidou, I., &
437 Teughels, W. (2021) A viability quantitative PCR dilemma: are longer amplicons better?
438 *Applied and Environmental Microbiology, 87*(5), e0265320.
439 https://doi.org/10.1128/aem.02653-20

440

441 Zhang, X., Ovenden, B., & Milgate, A. (2020) Recent insights into barley and *Rhynchosporium*
442 *commune* interactions. *Molecular Plant Pathology, 21*(8), 1111-1128.
443 https://doi.org/10.1111/mpp.12945

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449

450 **List of Figures**

451 **Figure 1**

452 Summarized workflow of the digital droplet PCR assay (Created with BioRender.com)

453

454 **Figure 2**

455 Digital droplet PCR assay, pathogen detected are indicated on x-axis and coloured horizontal
456 line is indicating the threshold for the assay. Grey droplet population below the threshold
457 line is negative droplets. Fluorescence intensity is indicated as relative fluorescence units
458 (RFU)(A) Triplex assay for the detection of *R. graminicola*, *R collo-cygni* (cp/µl) and *P. teres*
459 (cp/µl) (B) Form-specific assay for the detection of forms of *P. teres*, FAM channel detects *P.*
460 *teres f. teres* while HEX channel is to detect *P. teres f. maculata*. *P. teres* ITS assay was the
461 control (labelled with Cy5).

462

463 **Figure 3**

464 Linearity of the triplex assay for the detection of (A) *P. teres* (Internal transcribed Spacer
465 (ITS) gene), (B) *R. graminicola* (Cytochrome b (Cyt b) gene) and (C) *R. collo-cygni*
466 (Transcription elongation factor (tef) gene).

467

468 **Figure 4**

469 Specificity of the multiplex assays (A) Triplex assay for the detection of *R. collo-cygni*, R.
470 *graminicola* and *P. teres* challenged with a number of barley pathogens (B) *P. teres* form-
471 specific assay exposed to various barley pathogens.

472

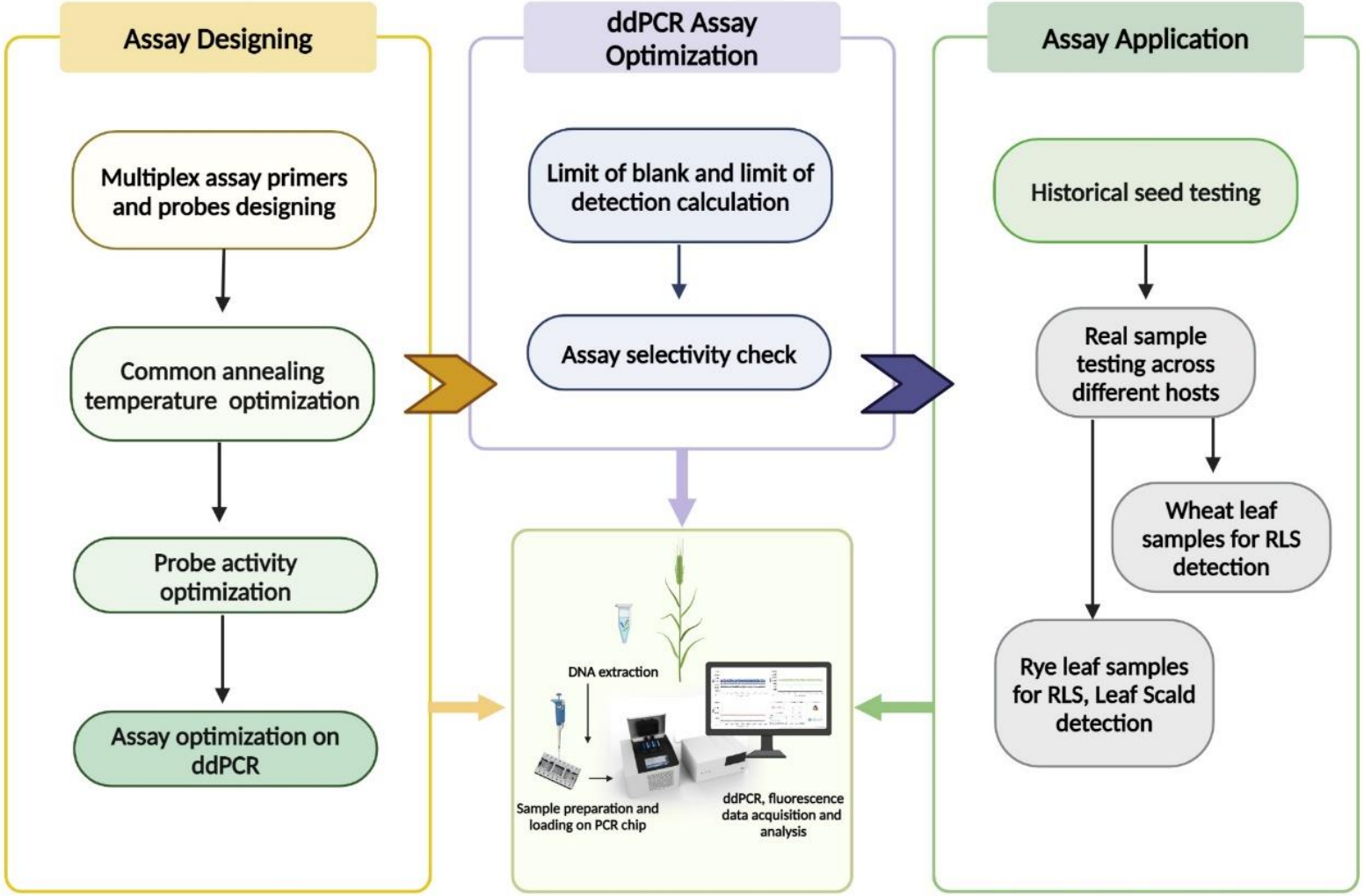
473 **Figure 5**

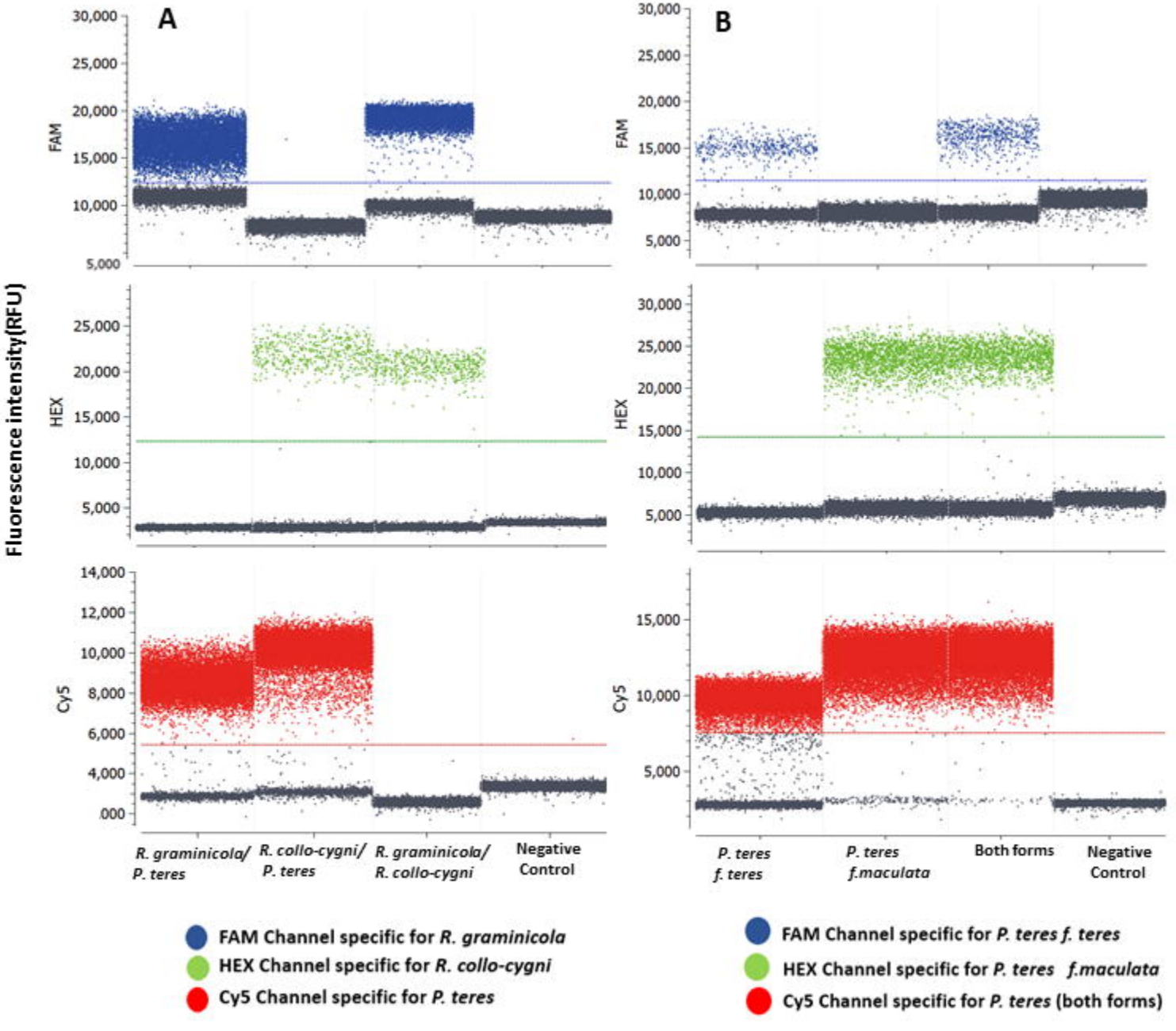
474

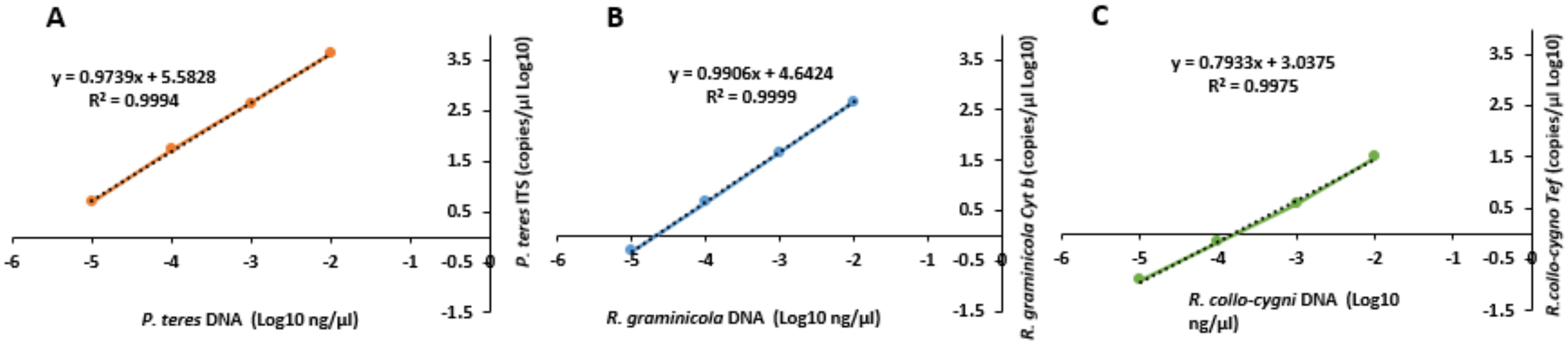
475 Detection of *R. collo-cygni*, *R. graminicola* and *P. teres* from wheat leaf DNA (sample 9, 11
476 and 13) collected from wheat volunteers in the barley field, sample 16 and 32 indicate
477 positive and negative controls for the triplex assay. Droplet population in FAM and Cy5
478 channel indicate the possible cross contamination of the wheat leaves with *R. graminicola*
479 and *P. teres* spores from the barley.

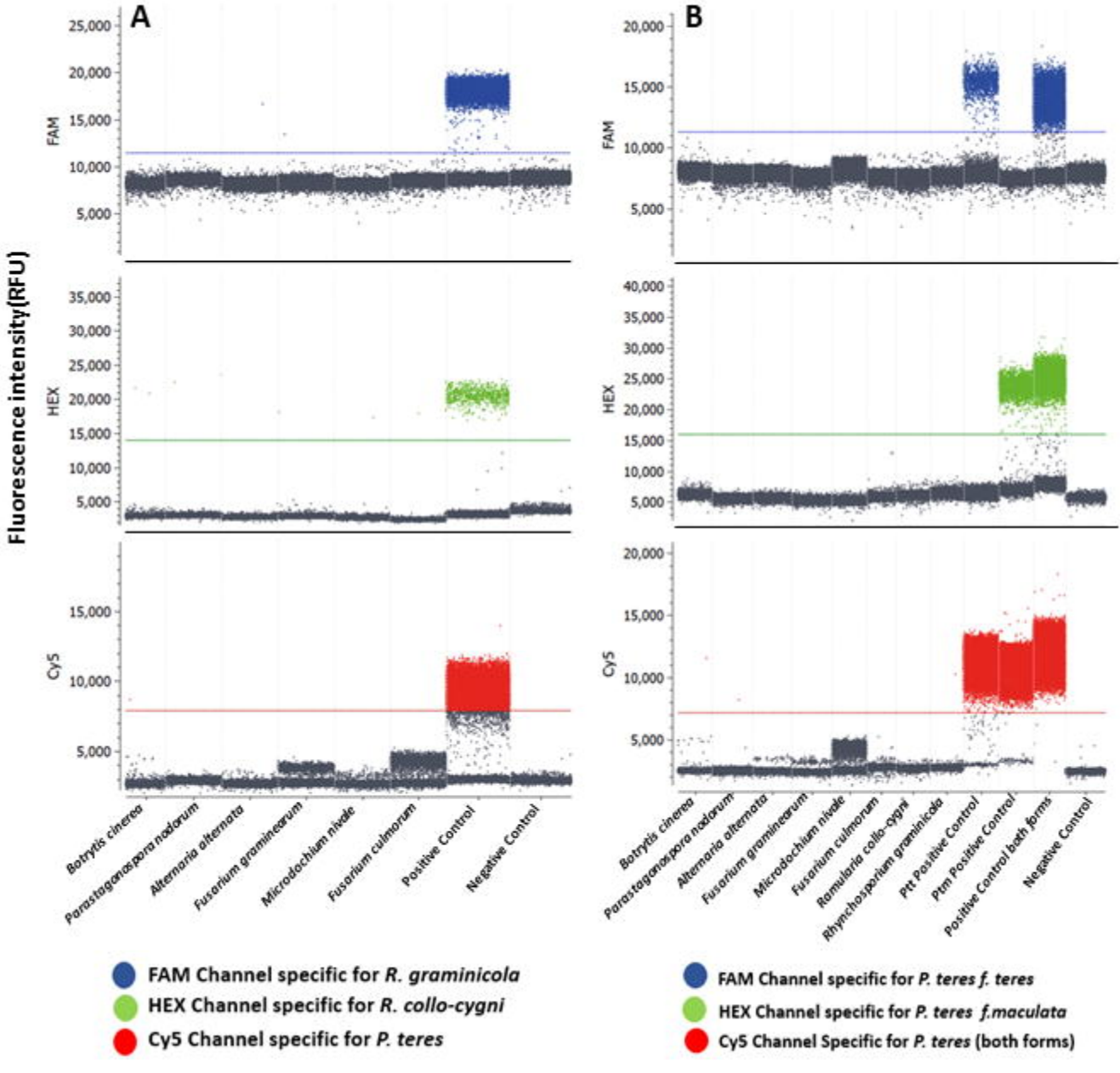
480

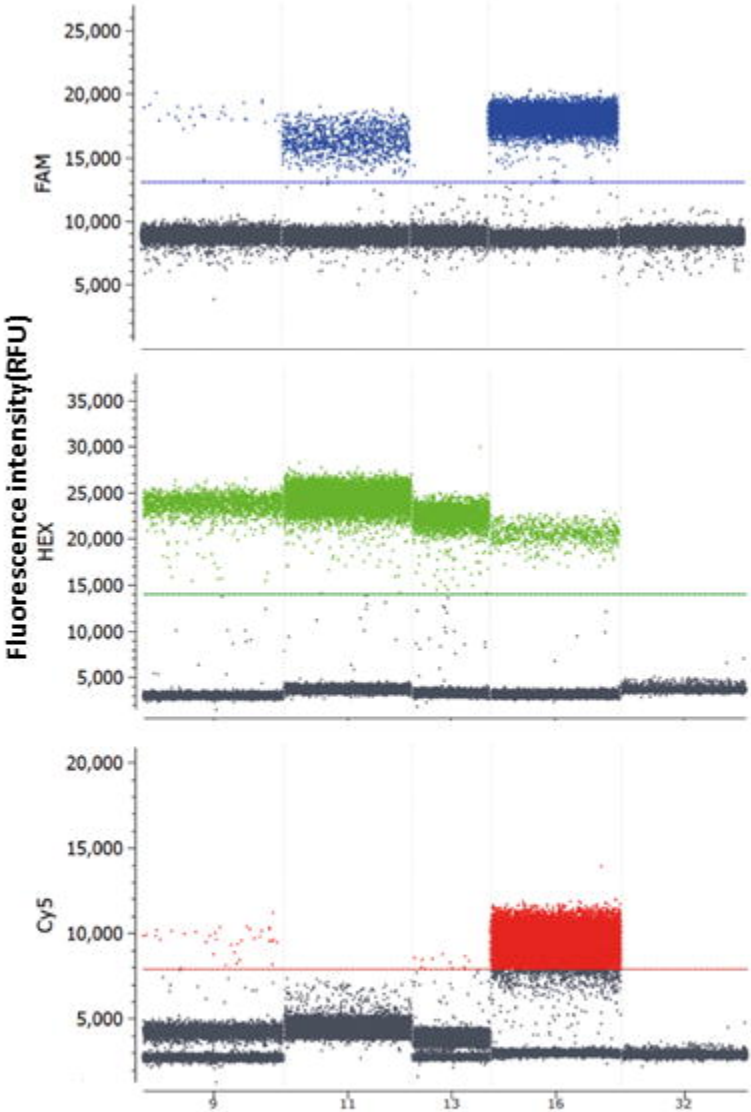
Figure 1


Assay Designing
Multiplex assay primers and probes designing
Common annealing temperature optimization
Probe activity optimization
Assay optimization on ddPCR
ddPCR Assay Optimization
Limit of blank and limit of detection calculation
Assay selectivity check
DNA extraction
Sample preparation and loading on PCR chip
ddPCR, fluorescence data acquisition and analysis
Assay Application
Historical seed testing
Real sample testing across different hosts
Wheat leaf samples for RLS detection
Rye leaf samples for RLS, Leaf Scald detection


A
B
Fluorescence intensity(RFU)
FAM
HEX
Cy5
R. graminicola/ P. teres
R. collo-cygni/ P. teres
R. graminicola/ R. collo-cygni
Negative Control
P. teres f. teres
P. teres f.maculata
Both forms
Negative Control
FAM Channel specific for R. graminicola
HEX Channel specific for R. collo-cygni
Cy5 Channel specific for P. teres
FAM Channel specific for P. teres f. teres
HEX Channel specific for P. teres f.maculata
Cy5 Channel specific for P. teres (both forms)


A
y = 0.9739x + 5.5828
R² = 0.9994
P. teres ITS (copies/µl Log10)
P. teres DNA (Log10 ng/µl)
B
y = 0.9906x + 4.6424
R² = 0.9999
R. graminicola Cyt b (copies/µl Log10)
R. graminicola DNA (Log10 ng/µl)
C
y = 0.7933x + 3.0375
R² = 0.9975
R.collo-cygno Tef (copies/µl Log10)
R. collo-cygni DNA (Log10 ng/µl)


A
B
Fluorescence intensity(RFU)
FAM
HEX
Cy5
Botrytis cinerea
Parastagonospora nodorum
Alternaria alternata
Fusarium graminearum
Microdochium nivale
Fusarium culmorum
Positive Control
Negative Control
Ramularia collo-cygni
Rhynchosporium graminicola
Ptt Positive Control
Ptm Positive Control
Positive Control both forms
FAM Channel specific for R. graminicola
HEX Channel specific for R. collo-cygni
Cy5 Channel specific for P. teres
FAM Channel specific for P. teres f. teres
HEX Channel specific for P. teres f.maculata
Cy5 Channel Specific for P. teres (both forms)



FAM Channel specific for *R. commune*

HEX Channel specific for *R. collo-cygni*

Cy5 Channel specific for *P. teres*

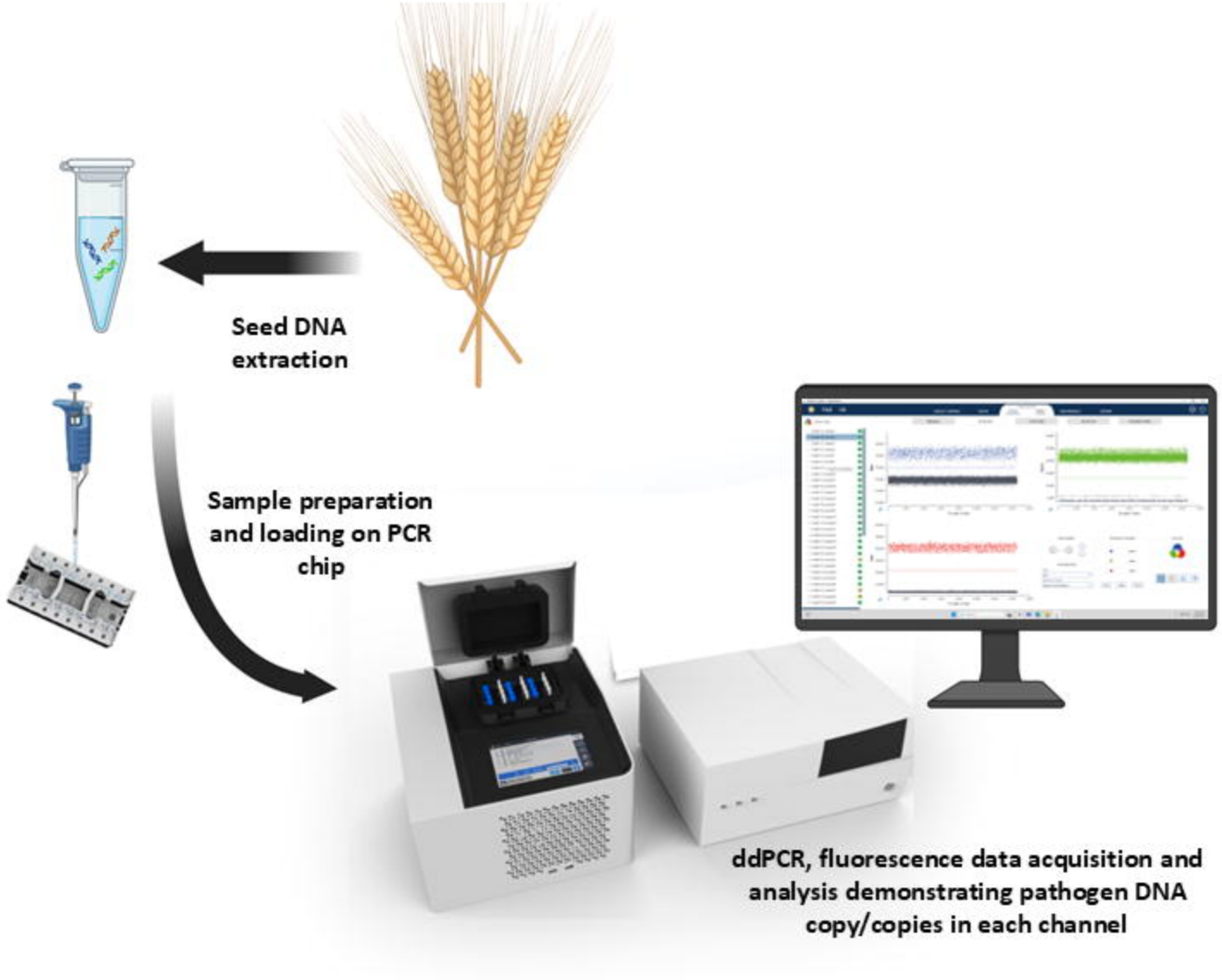

Seed DNA extraction
Sample preparation and loading on PCR chip
ddPCR, fluorescence data acquisition and analysis demonstrating pathogen DNA copy/copies in each channel

Table 1

List of primers and probes deployed in this study

Pathogen	Primer/Probe	Sequences (5' to 3')	Product size (bp)	Target gene
***P. teres* (Current study)**	Pyr_123 F*	TTCGAGCGTCATTTGTACCC	123	Internal transcribed spacer region
	Pyr_123 R**	CAAATAATGTGCTGCGCTCC		
	Pyr_123_P*** Cy5[a]	5Cy5/AAAACATTG/TAO/GCAGCCGGCCT/3IAbRQSp		
***R. collo-cygni* (Knight et al. 2022)**	Rcc_88_tef1-α_F	AGCCTAGCTGGCCACATGG	88	Transcription elongation factor
	Rcc_88_tef1-α_R	TGGGTGAGGTTGTCAGCATTT		
	Rcc_88_tef1-α_Pc HEX[b]	5HEX/ACATGCCGT/ZEN/CCAAATTTCCTCTACCATC /3IABkFQ		
***R. graminicola* Fountaine et al. 2007)**	Rsrtpcr1f	ATGTGCTTCCTTATGGACAGATGT	125	Cytochrome b
	Rsrtpcr1r	ATTATTAACAGAAAAACCCCCTCAGAT		
	Rsrtpcr1-Probe FAM[c]	56-FAM/TATG+AG+GTGCC+AC+AG/3IABkFQ		
***P. teres f. teres* (modified from Poudel et al. 2017)**	Ptt_173_F	GCATTGTCTAGCACTCGTCG	173	PttQ5 mRNA
	Ptt_173_R	CGCGGACTCAGAAGACATTG		
	Ptt_173_P FAM	56-FAM/CCTTCCGGT/ZEN/CAACAGTGGCTGTTA/3IABkFQ		
***P. teres f. maculata* (Modified from Poudel et al. 2017)**	Ptm_108_F	GATTAGACCATTACCACACTAGCG	108	PtmQ11 mRNA sequence
	Ptm_108_R	CGAGCAGTTCACGTTGTTCT		
	Ptm_108_P HEX	5HEX/TCGAGATTG/ZEN/CACACAGAACAAGGC/3IABkFQ		

*Forward primer

** Reverse primer

*** Probe sequence

a: Cyanine5 labelled probe, double quenched with TAO internal quencher, Iowa Black® RQ

b: Hexachlorofluorescein labelled probe, double quenched with ZEN internal quencher, Iowa Black FQ

c: 6-Carboxyfluorescein labelled probe, with 3' Iowa Black® FQ, + indicate locked nucleic acid (LNA) oligonucleotides