

# Scorpion ARMS primers for SNP real-time PCR detection and quantification of *Pyrenophora teres*

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## SUMMARY

We have developed a quantitative PCR detection method that can be used to determine the seed infection levels of *Pyrenophora teres*, a seed-borne fungal pathogen of barley. This method uses Scorpion Amplified Refractory Mutation System (ARMS) technology with real-time PCR detection. Scorpion ARMS primers were designed and optimized such that a single nucleotide base mismatch in the primer sequence could distinguish *P. teres* from *P. graminea*, a closely related seed-borne pathogen of barley. It is necessary to distinguish between these two agriculturally important pathogens since different disease management decisions are made, based on the presence and level of infection measured for each. The advance in development of sensitive and specific fluorescent probes has enabled the current PCR test to detect *Pyrenophora* spp. pathogenic on barley to be enhanced with the advantage that it can now specifically detect *P. teres* in a single reaction, whilst previously, two reactions were required to discriminate *P. teres* from *P. graminea*.

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## INTRODUCTION

Scorpion primers combine a probe with a specific target sequence for PCR in a single molecule, resulting in a fluorescent detection system with unimolecular kinetics (Thelwell *et al.*, 2000). This has the advantage over other fluorescent probe systems such as Molecular Beacons and TaqMan, in that no separate probe is required to bind to the amplified target, making detection both faster and more efficient. A direct comparison of the three detection methods (Thelwell *et al.*, 2000) indicates that Scorpions perform better than intermolecular probing systems, particularly under rapid cycling conditions.

The structure of a Scorpion primer is such that it is held in a hairpin loop conformation by complementary stem sequences of around six bases which flank a probe sequence specific for the

target of interest (Whitcombe *et al.*, 1999). The stem also serves to position together a fluorescent reporter dye (attached to the 5'-end) in close proximity with a quencher molecule. In this conformation, no signal is produced. A PCR-blocker separates the hairpin loop from the primer sequence, which forms the 3'-end of the Scorpion. The blocker prevents read-through, which would lead to unfolding of the hairpin loop in the absence of a specific target. During PCR, extension occurs as usual from the primer. After the subsequent denaturation and annealing steps, the hairpin loop unfolds and, if the correct product has been amplified, the probe sequence binds to the specific target sequence downstream of the primer on the newly synthesized strand. This new structure is thermodynamically more stable than the original hairpin loop. A fluorescent signal is now generated, since the fluorescent dye is no longer in close proximity to the quencher.

Scorpions can be used in combination with the Amplification Refractory Mutation System (ARMS) (Newton *et al.*, 1989; Whitcombe *et al.*, 1999) to enable single base mutations to be detected. Under the appropriate PCR conditions a single base mismatch located at the 3'-end of the primer is sufficient for preferential amplification of the perfectly matched allele (Newton *et al.*, 1989), allowing the discrimination of closely related species.

There are two *Pyrenophora* spp. which are important barley pathogens. *Pyrenophora teres* Drechs. and *Pyrenophora graminea* Ito & Kuribay. *P. teres* causes net blotch on barley, which is responsible for yield loss and reduction in malting quality in most barley-growing areas of the world (Weiland *et al.*, 1999). It is predominantly seed-borne but crop debris can also be a source of inoculum (Taylor *et al.*, 2001). *P. graminea* causes leaf stripe on barley which results in the failure of the plant to produce ears. This disease is strictly seed-borne, and so infection levels may increase rapidly in successive generations of untreated seed (Thomas *et al.*, 1998). At present in the UK, chemical seed treatments are used to control *P. graminea* and *P. teres* when barley seed infection levels are found to be 2% or more. Foliar fungicide sprays are also effective at controlling *P. teres* if plants become infected after emergence.

Due to the narrow window of time between harvest and sowing, it is important to have a rapid, quantitative seed test to enable the farmer to make the appropriate disease management decision for

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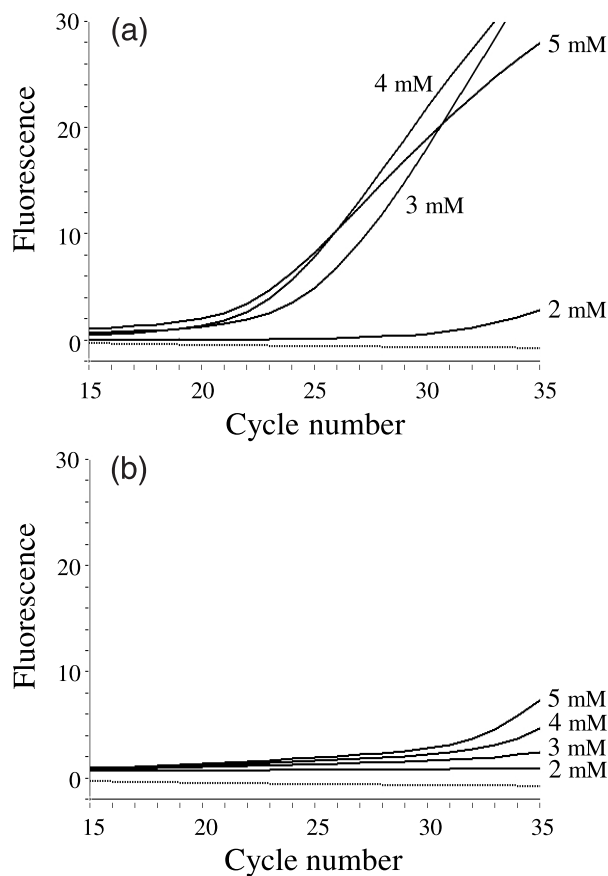
chemical treatments. Voluntary or advisory seed health tests based on microscopic analysis to detect *Pyrenophora* spp. on barley seed are labour intensive and slow, taking seven or more days to complete. A new test that can be completed in a day has been developed and run as a commercial service at NIAB during the last year (Bates *et al.*, 2001; Taylor *et al.*, 2001). This PCR test is performed in two stages: (i) quantitative real-time PCR with generic *Pyrenophora* spp. primers based on 5.8S rRNA internal transcribed spacer (ITS) sequences (Stevens *et al.*, 1998), and (ii) a specific PCR test performed on positives from (i) to check for the presence of *P. graminea*. The PCR test is performed on a Light-Cycler instrument, which is capable of real-time PCR detection with in-tube product analysis. This minimizes the chances of cross-contamination and also has the added advantage of being nearly 10 times faster than a conventional PCR machine. We describe here the development of a Scorpion ARMS primer for the specific detection and quantification of *P. teres*, which can distinguish a single nucleotide difference between the two species.

## RESULTS AND DISCUSSION

### Optimization of the *P. teres* Scorpion ARMS PCR assay

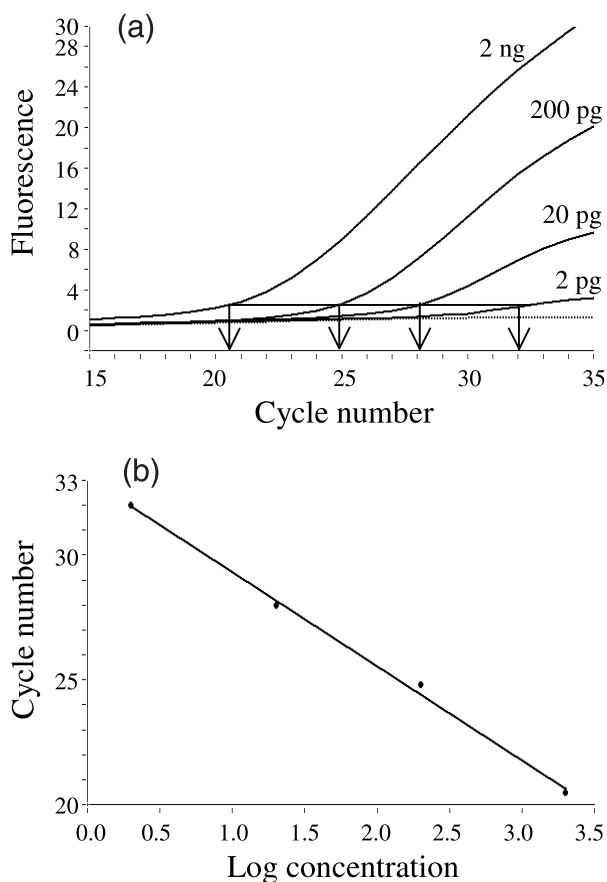
Conventionally, Scorpion primers are designed with the specific diagnostic sequence contained within the probe region of the oligonucleotide (Thelwell *et al.*, 2000). In this instance, primer extension might occur from non-target DNA, but the product will not be detected if there is a mismatch between the product and probe sequences. However with Scorpion ARMS primers, the primer is sited over the polymorphic site rather than the probe (Whitcombe *et al.*, 1999). In this case, under optimal reaction conditions, primer extension will only occur from the target DNA to which the primer has been designed, as in conventional PCRs. Single base mismatches at the 3'-end can be distinguished under appropriate conditions, or additional mismatches introduced to enhance specificity (Whitcombe *et al.*, 1999). The probe section of the Scorpion can be used to further increase the specificity of the reaction if there are additional sequence differences downstream of the primer within the product. The high degree of secondary structure in the *P. teres* ITS1 sequence, together with only a single base pair difference in this region between *P. teres* and *P. graminea*, made the design of a conventional Scorpion difficult. In addition, for design of TaqMan and Molecular Beacons probes, PCR primers also have to be taken into account which constrains the design and could potentially increase non-specific interactions. However, a Scorpion ARMS primer was successfully developed for the detection of *P. teres* by exploiting the single base pair difference at position 42 of the ITS1 sequence (Stevens *et al.*, 1998), placing this at the 3'-end of the primer sequence.

Both *P. teres* and *P. graminea* DNA samples (2 ng) were run in parallel reactions during optimization to test the specificity of the



**Fig. 1** Optimization of the *P. teres* Scorpion assay by MgCl<sub>2</sub> titration. (a) *P. teres* DNA and (b) *P. graminea* DNA. 2 ng template was used in each reaction. mM are concentrations of MgCl<sub>2</sub> in the PCR. The no-template control (with 4 mM MgCl<sub>2</sub>) is shown as a dotted line.

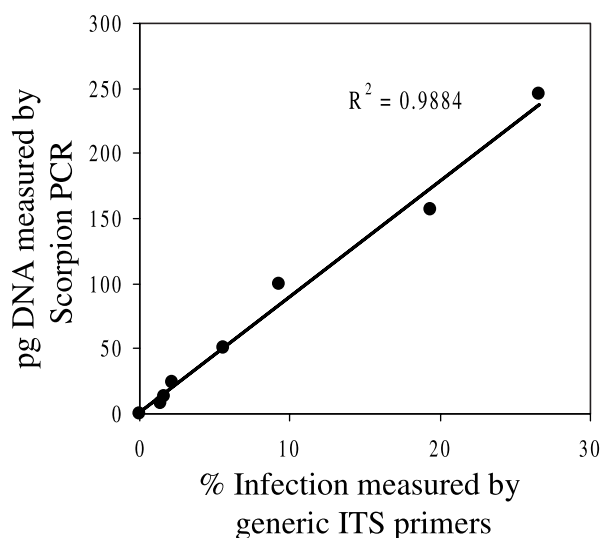
*P. teres* Scorpion primer. Of the four MgCl<sub>2</sub> concentrations tested, 4 mM gave an optimal product amplification from *P. teres* (Fig. 1a). Some *P. graminea* did appear to amplify near the end of the cycling reactions as the MgCl<sub>2</sub> concentrations were increased (Fig. 1b), however, this could be avoided by reducing the number of amplification cycles to 33. The good correlation between the PCR results with the Scorpion primers, and previous work with ITS1 generic primers (Bates *et al.*, 2001) has indicated that products amplified from 2 ng DNA represents at least 70% of the seed infected with *P. teres*, and would be similar for *P. graminea*. In practical terms, a seed sample tested using this assay would have to be highly infected ( $\geq 70\%$ ) with *P. graminea* to cause a false positive result. This seed lot would require treatment in any case. This situation is very unlikely to arise since levels of infection with *P. graminea* have dropped to virtually zero over the last 5 years in seed samples submitted to the Official Seed Testing Station (OSTS), Cambridge, UK, for advisory testing (Thomas *et al.*, 2000).



**Fig. 2** Quantification of *P. teres* DNA using Scorpion primers. (a) Serial dilution of *P. teres* DNA; values shown on the chart are pg DNA included in the PCR. (b) A standard curve for calculation of the DNA concentration of unknowns can be derived by plotting the crossing point (cycle number) of each curve at an arbitrarily set point within the log-linear stage of amplification (shown in (a)), against the log of the standard DNA concentration.

### Specificity of the *P. teres* Scorpion ARMS PCR assay

The specificity of the Scorpion primer was checked by screening a number of different *Pyrenophora* isolates and other pathogens and saprophytes commonly found on barley (Table 1). The Scorpion primer amplified all the *P. teres* isolates tested, which were obtained from geographically diverse locations. As expected, the Scorpion primers also amplified the two isolates of *P. teres* f.sp. *maculata*, a subspecies of *P. teres* which also has a T residue at position 42 of the ITS sequence (Stevens *et al.*, 1998) in common with *P. teres*. The subspecies, *P. teres* f.sp. *maculata* is not commonly reported in the UK but is observed with greater frequency in Scandinavian countries. Disease management of *P. teres* f.sp. *maculata* is similar to that of *P. teres* and so it is not necessary to distinguish this pathogen in a seed health test. The only other species to amplify with the *P. teres* Scorpion primer were isolates of *P. hordei*, which also have a T residue at position 42 of the ITS1



**Fig. 3** Correlation between percentage infection levels of seed samples measured by PCR test with generic *Pyrenophora* spp. primers (Bates *et al.*, 2001) and quantification using the Scorpion primers. Eight barley seed samples ranging from 0% to 26.6% *P. teres* infection were tested.  $n = 4$  for 19.4% sample;  $n = 3$  for 26.6% sample. All others are single Scorpion PCRs.

sequence (Stevens *et al.*, 1998). *P. hordei* has recently been reclassified as *P. teres* f.sp. *maculata*, which explains the positive result (Williams *et al.*, 2001).

### Test sensitivity and quantification of *P. teres* DNA

We have previously described the quantification of *P. teres* DNA in seed extracts using SYBR Green I (a product-independent fluorescent dye) and the generic primers ITSFF and ITSr (Bates *et al.*, 2001). However, these primers are specific for any *Pyrenophora* species that infect barley and thus will also amplify any *P. graminea* present in the sample. Using the Scorpion primer set, quantification of *P. teres* alone can be achieved—with 2 pg *P. teres* DNA readily detected after 35 cycles of amplification (Fig. 2a). The fluorescence gain is considerably decreased compared with SYBR Green I, since only one fluorescent signal is detected per amplicon, whereas SYBR Green I intercalates with the double stranded product giving larger but nonspecific signals. However, specificity is increased since no *P. graminea* is amplified. Amplification of around 2 pg *P. teres* DNA represents detection of levels of infection well below the 2% UK voluntary threshold level for application of fungicide treatments (Bates *et al.*, 2001). The DNA extraction method developed for this seed health test (Taylor *et al.*, 2001) effectively removed PCR inhibitors present in barley seed. This is important for accurate PCR quantification in a Lightcycler as the presence of inhibitors would affect the accuracy and reproducibility of the results. Replicate samples produced no significant difference in PCR results (Fig. 3). This

**Table 1** Details of the *Pyrenophora* spp. and other fungal isolates tested.

Isolate identity	Host source	Country of origin	Date of isolation	Amplification with the <i>P. teres</i> Scorpion primer
Pt 93-9	Barley	UK	1993	+
Pt 93-10	Barley	UK	1993	+
Pt 93-20	Barley	UK	1993	+
Pt 93-22	Barley	UK	1993	+
Pt 93-34	Barley	Argentina	1993	+
Pt 93-35	Barley	Argentina	1993	+
Pt 95-17	Barley	UK	1995	+
Pt 95-32	Barley	Sweden	1995	+
Pt 95-60	Barley	Norway	1995	+
Pt 96-1	Barley	Russia	1996	+
Pt 96-2	Barley	Russia	1996	+
Pt 96-3	Barley	Russia	1996	+
Pt 96-6	Barley	Russia	1996	+
Pt 97-78d	Barley	Russia	1997	+
Pt 97-78f	Barley	Russia	1997	+
Pt 97-78g	Barley	Russia	1997	+
Pt 97-BS67/2	Barley	France	1997	+
Pt 97-BS67/4	Barley	France	1997	+
Pt 97-BS67/5	Barley	France	1997	+
Pg 95-5	Barley	Denmark	1995	-
Pg 97-BS1	Barley	n/a	1997	-
Pg 97-BS6-1	Barley	Yugoslavia	1997	-
Pg 97-76a	Barley	UK	1997	-
Pg 97-78a	Barley	UK	1997	-
Pg 97-78c	Barley	UK	1997	-
Pg 97-81b	Barley	Italy	1997	-
Pg 97-81d	Barley	Italy	1997	-
Pg 97-81g	Barley	Italy	1997	-
Pg 97-83a	Barley	UK	1997	-
Pg 97-83b	Barley	UK	1997	-
Pg 97-83c	Barley	UK	1997	-
Pg 97-83d	Barley	UK	1997	-
Pg 97-83f	Barley	UK	1997	-
Pg 97-83g	Barley	UK	1997	-
Pg 97-83h	Barley	UK	1997	-
Pg 97-83j	Barley	UK	1997	-
Pg 97-83k	Barley	UK	1997	-
Pg 97-83l	Barley	UK	1997	-
Pg BS4-8	Barley	Italy	1998	-
Pm 857	Barley	Canada	1996	+
Pm 1881a	Barley	Canada	1996	+
<i>P. avenae</i> 94-1	Oats	UK	1994	-
<i>P. avenae</i> 94-3	Oats	UK	1994	-
<i>P. hordei</i> 94-1	Barley	Australia	1994	+
<i>P. hordei</i> 94-12	Barley	Australia	1994	+
<i>P. bromi</i> 94-1	Brome	Denmark	1994	-
<i>P. lolii</i> 95-1	Ryegrass	UK	1995	-
<i>Alternaria tenuis</i> 97-1	n/a	UK	1997	-
<i>Cochliobolus sativus</i> 97-10	Wheat	UK	1997	-
<i>Epicoccum</i> spp. 97-1	n/a	UK	1997	-
<i>Fusarium culmorum</i> 98-11	Wheat	UK	1998	-
<i>Fusarium moniliforme</i> 98-1	n/a	n/a	1998	-
<i>Fusarium oxysporum</i> 98-1	Linseed	UK	1997	-

Table 1 continued.

Isolate identity	Host source	Country of origin	Date of isolation	Amplification with the <i>P. teres</i> Scorpion primer
<i>Fusarium poae</i> 94-1	Wheat	UK	1995	–
<i>Fusarium</i> spp. 97-1	Bean	UK	1997	–
<i>Microdochium nivale</i> 98-26	Wheat	UK	1998	–
<i>Septoria nodorum</i> 98-2	Wheat	UK	1998	–
<i>Tilletia caries</i> 00-1	Wheat	UK	2000	–
Barley var. Alexis	–	UK	1997	–
Barley var. Triumph	–	UK	1997	–

Abbreviations: Pt = *P. teres*, Pg = *P. graminea*, Pm = *P. teres* f. sp. *maculata*, + = positive reaction, – = negative reaction, n/a = information not available.

demonstrated that the test was reproducible using Scorpion primers and the results compared well with the existing PCR test when used to quantify unknown test samples (Fig. 3). As with the existing PCR test, it is possible to convert the DNA quantification result to a percentage infection result by correlation of the PCR test with agar test results. This is important data for validating the use of Scorpion primers in routine seed health testing for detection of *P. teres*, which has a voluntary 2% infection threshold. A recent paper (Williams *et al.*, 2001) has described a PCR test that can discriminate between the two forms of barley net blotch (*P. teres* f. sp. *maculata* and *P. teres* f. sp. *teres*). However, the assay showed a cross-reaction with *P. graminea* and could not differentiate these two species and thus cannot be used for seed health testing.

The Scorpion ARMS primer developed in this study allowed a single point mutation to be distinguished, even when the design of alternative molecular probes, such as Molecular Beacons and Taqman, was not possible due to the high degree of secondary structure surrounding the mismatch. The Scorpion primer was also useful for the calculation of disease infection levels by quantification of target template DNA amplification. The fluorescence signal produced by the Scorpion probe can only be generated if the target sequence has been amplified, allowing the probe to hybridize with newly synthesized target. This gives the reaction a high level of specificity and sensitivity. The PCR test described is specific, sensitive, reproducible and rapid, making it an excellent system for screening large numbers of seed samples.

## MATERIALS AND METHODS

### Origin and maintenance of fungal isolates

The *Pyrenophora* spp. and other fungal isolates used in this study were obtained from geographically diverse areas and host species (Table 1). Fungal isolates derived from single conidia were identified, cultivated and maintained as described by Stevens *et al.* (1998).

### DNA extraction from fungal cultures and *P. teres* infected seed samples

Genomic DNA was extracted from fungal cultures as described previously (Taylor *et al.*, 2001). Fungal DNA from infected seed submitted to the OSTs, Cambridge, UK for advisory testing was extracted as described by Bates *et al.* (2001). Samples that had previously tested positive for *P. teres* infection by PCR test (Bates *et al.*, 2001) were used in this study.

### Development and optimization of the Scorpion ARMS PCR assay

The Scorpion primer specific for *P. teres* was based on *Pyrenophora* ITS1 sequences (Stevens *et al.*, 1998) and designed and synthesized by Oswel Research Products Ltd (Southampton, UK). The oligonucleotide consists of a hairpin loop structure (Whitcombe *et al.*, 1999) with a 5'-fluorescein (FAM) dye, internal quencher (MR = methyl red), PCR blocker (HEG = hexaethylene glycol), 3'-PCR primer sequence (underlined) and a probe sequence within the loop (in bold): 5'-FAM-CCGCGG**GCGCCA-GAATGGGCAAA**CCGCGG-MR-HEG-TGGGTAGTCCCGCTTTT-3'. The Scorpion is held in a hairpin loop configuration by the complementary stem sequences at either end of the probe sequence. The primer and probe sequences correspond, respectively, to bases 21–42 and 48–73 (reverse and complement) of the *P. teres* alignment given in Stevens *et al.* (1998), the specificity of the primer being determined by a 3'-terminal T residue at position 42 (*P. graminea* has a G residue in this position). The primer primes off the antisense strand of the target and the probe binds newly synthesized sense strand DNA. The primer was purified by double HPLC. The reverse primer used together in the PCR with the Scorpion primer corresponds to bases 172–193: 5'-ATTGATTACATTGTTTGCTGA-3' (reverse and complement).

All PCRs were performed in a LightCycler instrument (Roche Diagnostics Limited, Lewes, UK). Reaction mixtures consisted of 1 × FastStart Hybridization Probes Master Mix (Roche Diagnostics, Cat. no. 3003248); 0.5 μM of each primer; MgCl<sub>2</sub> to

the required concentration (2, 3, 4 or 5 mM); 2 µL of DNA template (2 ng unless otherwise specified) and water to a volume of 20 µL. Reaction mixes were loaded into chilled glass capillary tubes (Roche Diagnostics, Cat. no. 1909339) by centrifugation at 700 g for 5 s.

For the quantification assay, a serial dilution of *P. teres* genomic DNA was prepared. DNA concentration was determined using a fluorescent assay in a DyNA Quant 200 fluorimeter (Hoefer Pharmacia Biotech Inc., USA) using the Hoescht 33258 dye. *P. teres* standards were then prepared by dilution to 1 ng/µL, 100 pg/µL, 10 pg/µL and 1 pg/µL.

For the Scorpion PCRs the cycling programme was as follows: initial denaturation at 95 °C for 10 min (to activate the 'hot start' *Taq* polymerase), followed by 35 cycles of 95 °C for 0 s, 63 °C for 5 s, 45 °C for 5 s and 72 °C for 10 s. PCR product accumulation was measured once during each amplification cycle at the end of the 63 °C annealing step on channel 1. For quantification, a standard curve was generated by plotting the log of the DNA concentration of the known standards against the cycle number at a defined point in the log-linear increase in the fluorescence of the PCR product (crossing point) (see Fig. 2).

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