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Synthetic Internal Control Sequences to Increase Negative Call Veracity in Multiplexed, Quantitative PCR Assays for *Phakopsora pachyrhizi*

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Germinating Urediniospore & Germination Tube of *P. pachyrhizi*

INTRODUCTION & UTILITY

Negative results frequently do not generate the sensational impact of positives, and as such may not as often elicit the "prove it" response. Crop security, conscientious disease management, and commercial liability do demand confidence in the validity of a negative result. Unfortunately, many researchers and diagnostic labs may not have an available internal control to reveal false-negatives.

False-negatives can be produced by diagnostic Q-PCR assays for multiple reasons: operator error during reaction assembly, physical component failure (leaking reaction tube, wear and damage to fiber optics and moving parts, etc.), or inhibitors to the PCR chemistry carried through (or introduced by) the DNA isolation process (chelating agents, detergents, proteases, phenolics, particulates & immobilants, etc).

We here demonstrate the function of two artificial DNA internal control target molecules (with their matching primers and probes) to cull false-negative calls in TaqMan assays by producing a positive signal to validate the PCR process within each individual reaction.

PpaIC is a single-stranded oligonucleotide internal control, arranged as a core sequence (Figure 1) flanked by sequences complementary to the primers of the *P. pachyrhizi* assay itself, affording simplicity in reaction assembly. We have successfully replaced the flanking primer sequences with those specific for *P. meibomiae*, as well as for several other fungi for which we have TaqMan assays—and the same Cy5 probe can be used for all, thus reducing reagent costs.

HHIC is a double-stranded, general-purpose DNA target. It utilizes independent primers and probe, has proven useful when multiplexed into a variety of Taqman assays, and is not limited to any specific primary target. It could also be used with bacterial, viral, protozoan, animal and plant Taqman assays. Because it is a dsDNA, the linearized plasmid may also be spiked into raw samples as a tracer to estimate purification yields, etc.

DEVELOPMENT

PpmIC: An 87 nt target oligonucleotide incorporating an artificial core sequence of 42 nt, flanked by primer-binding sequences complementary to the primers Ppm1 & Ppa2, and a 25 nt long Cy5-labeled and IAB-quenched probe, Ppm-IC-p (Figure 1).

HHIC: A 111 nt completely artificial insert sequence (Figure 2) cloned and transformed into *E. coli* for production. The resulting 3,480 nt plasmid was purified, linearized, and quantified by Poisson analysis. A 23 nt HEX-labeled and IAB-quenched probe anneals between flanking PCR primer sites within the artificial sequence (Figure 2). The residual plasmid provides stability to the dsDNA.

Q-PCR conditions: Internal control target molecules were diluted to a 500x working stock of 1 fM; 50 ymole were added per 25 µL Q-PCR reaction. Other reaction components were assembled at the final concentrations shown in Table 1. Both PpmIC and HHIC have been run simultaneously with the standard *P. pachyrhizi* assay (using SBR-FAM probe) and ROX reference dye, as a quadriplex reaction. Primers for *P. pachyrhizi* are, by necessity, at the concentrations optimized for that assay, while HHIC primers and both probes are used at empirically-determined optimal concentrations. Invitrogen Platinum qPCR Supermix-UDG was used to achieve amplification in a Stratagene MX3005P real-time thermal cycler with the following regimen: 2 min at 60°; 2 min at 95°; 40 cycles of [15 sec at 95°; 30 sec at 60°].

Figure 1.
Nucleotide sequence of the PpmIC oligo, as a double-stranded amplicon. Primer (green) and probe (red) binding sites are indicated. The 42 nt core sequence is indicated (blue box).

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5′-GCAGAATTCA GTGAATCATC AAG CATGCTT AGGACGAGAA CTCCCACATC GAGCTGGACA TCTGC ATTGT TGGATTTTGA GTGTTGC-3′
3′-CGTCTTAAGT CACTTAGTAG TTC GTACGAA TCCTGCTCTT GAGGGTGTAG CTCGACCTGT AGTCG TAACA ACCTAAAACT CACAACG-5′
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Sequence of the (25 nt) probe "Ppm-IC-p": 5′-TGCTTAGGACGAGAACTCCCACATC-3′

Sequence of the (23 nt) primer† "Ppm1": 5′-GCAGAATTCAGTGAATCATCAAG-3′

Sequence of the (22 nt) primer† "Ppa2": 5′-GCAACACTCAAAATCCAACAAT-3′

†These two primer sequences are included in United States Patent 7097975, and published by Frederick, *et. al*, 2002 Phytopathology 92(2):217-227.

Figure 2.
Nucleotide sequence of the artificial insert contained by the plasmid target HHIC. Primer (green) and probe (red) binding sites are indicated:

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5′-CAATCAGCGG GTGTTTCAGC ACAAGGCGTT GCGCTCGGTG TTGATGTTTG CCATGGTCCT GGCGTGGTCG TTTGCGTCAA GCTCGATGTG GGAGTTCTCG TCCTAGGCGT G-3′
3′-GTTACTCGCC CACAAAGTCG TGTTCCGCAA CGCGAGCCAC AACTACAAAC GGTACCAGGA CCGCACCAGC AAACGCAGTT CGAGCTACAC CCTCAAGAGC AGGATCCGCA C-5′
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Sequence of the (23 nt) probe HHIC-p: 5′-TCGGTGTTGATGTTTGCCATGGT -3′

Sequence of the (18 nt) primer driving probe hydrolysis, HHIC-rev: 5′-CAATCAGCGGGTGTTTCA -3′

Sequence of the (21 nt) primer for amplicon synthesis in the opposite direction, HHIC-fwd: 5′-CTAGGACGAGAACTCCCACAT-3′

Table 1. Reagents used in 25 µL reactions.

Component	Final Concentration
MgCl2	7.0 mM
Primer Ppm1	300 nM
Primer Ppa2	300 nM
Probe SBR-FAM	100 nM
Probe Ppm-IC-p (Cy5)	150 nM
Primer HHIC-fwd	600 nM
Primer HHIC-rev	200 nM
Probe HHIC-p (HEX)	200 nM
ROX dye	50 nM
PpmIC oligo target	30 copies
HHIC plasmid target	30 copies
Primary target DNA	variable

Figure 3

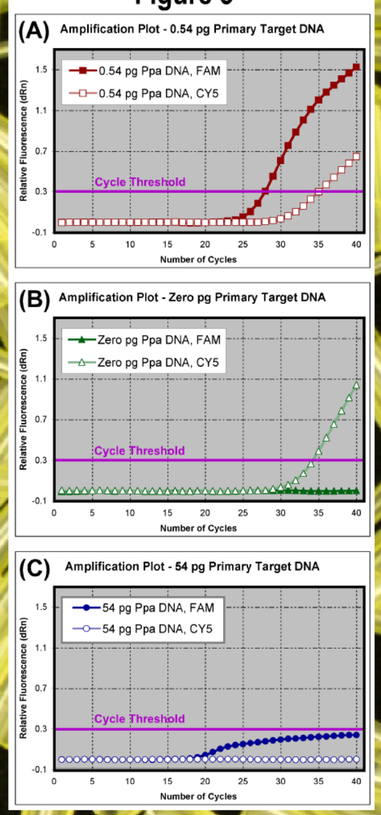
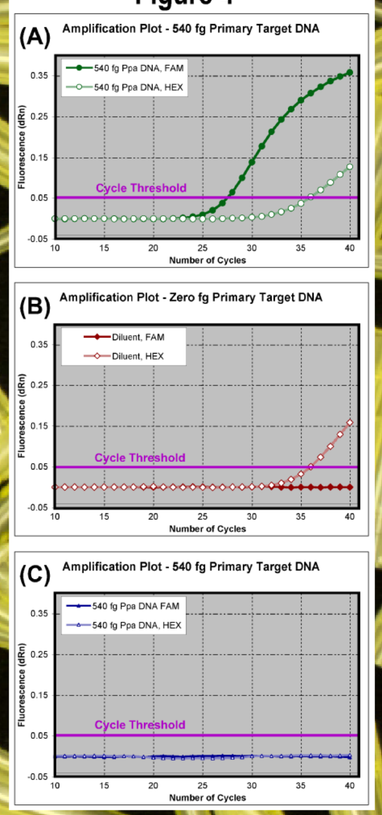


Figure 4



Figures 3 and 4.
Functionality of the PpaIC and HHIC molecules in actual Q-PCR assays for *P. pachyrhizi*.

(A): A positive Q-PCR reaction progresses, with increasing FAM signal produced from the primary target (0.54 pg *P. pachyrhizi* DNA). The FAM fluorescence signal crossed the cycle threshold and at the end of the reaction was scored as positive. Similarly, Cy5 (Fig.3) and HEX (Fig. 4) signals increased, indicating that their respective IC targets were amplified.

(B): A true-negative Q-PCR reaction progresses in a tube with no primary target DNA (diluent only). FAM fluorescence remained below the cycle threshold and at the end of the reaction was scored as negative. Because the Cy5 (Fig. 3) and HEX (Fig. 4) signals from their respective IC targets were amplified, the PCR chemistry was active and had any primary target DNA been present, it would have been detected; thus these reactions are validated as true negatives.

(C): A questionable Q-PCR reaction progresses in the presence of primary target (54 pg *P. pachyrhizi* DNA in Fig. 3 and 0.54 pg *P. pachyrhizi* DNA in Fig. 4). The FAM fluorescence remained below the cycle threshold, and at the end of the reaction was scored as negative. However, the Cy5 (Fig. 3) and HEX (Fig. 4) signals also remained below the cycle threshold, indicating that amplification of their respective IC molecules did not occur, and therefore the reactions were faulty in some way. Thus, the results were re-scored as false-negatives, and excluded from further consideration.

ABSTRACT

Quantitative PCR (Q-PCR) utilizing specific primer sequences and a fluorogenic, 5'-exonuclease linear hydrolysis probe is well established as a detection and identification method for *Phakopsora pachyrhizi*, the soybean rust pathogen. Because of the extreme sensitivity of Q-PCR, the DNA of a single urediniospore of this fungus can be detected from total DNA extracts of environmental samples. However, some DNA preparations unpredictably contain PCR inhibitors that increase the frequency of false-negatives indistinguishable from true-negatives. Two synthetic DNA molecules of artificial and arbitrary sequence were constructed, and their functionality demonstrated with the matching primers and probes as multiplexed, internal controls (ICs), to cull false-negative results by producing a positive signal that validates the PCR process within each individual reaction. The first, PpaIC, is a single-stranded oligonucleotide flanked by sequences complementary to the primers of the *P. pachyrhizi* assay itself, but targeted by a unique probe. The second, HHIC, is a dsDNA designed to utilize unique primers and probe, and was prepared as a cloned sequence in a linearized plasmid. Either IC may be added in trace amounts to raw samples before DNA extraction, or to reagent mixtures during assay set-up. Neither IC has significant similarity to natural sequences present in public databases and, whereas PpaIC will be useful mainly in *P. pachyrhizi* testing, HHIC has potential for use in a broad range of multiplexed Q-PCR assays when a natural internal control against false-negatives is unavailable.

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