

Justification for Removal of the ZUP (IS1002) qPCR Assay from the SE-qPCR Protocol for *Paracidovorax citrulli* Detection in Cucurbit Seeds

Addendum Validation report, October 2025

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Summary

The ZUP qPCR assay, currently one of three options in the ISHI SE-qPCR protocol for *Paracidovorax citrulli* detection, has been shown to produce false positives due to its poor specificity. Recent genomic and diagnostic findings highlight the risks of relying on the ZUP qPCR assay, even in combination with other qPCR assays. This document presents the rationale for its removal from the current ISHI protocol (ISHI, 2021) based on both experimental data and recent field observations.

Introduction

The current ISHI protocol for the detection of *Paracidovorax citrulli* (formerly known as *Acidovorax citrulli*) in cucurbit seeds by SE-qPCR (v2.0, May 2021) allows laboratories to use a combination of any two of the three validated qPCR assays: ZUP, Contig 21, and Contig 22 (ISHI, 2021).

Based on the validation data presented in the ISHI report (Kleinhesselink, 2017), the ZUP (IS1002) qPCR assay shows significantly reduced specificity compared to the Contig 21 and Contig 22 assays. While all three qPCR assays detect 100% of *P. citrulli* isolates, the ZUP qPCR assay cross-reacted with 35% of non-*P. citrulli* isolates, in contrast to 0% for Contig 21 and 4% for Contig 22 (Table 6 from Kleinhesselink, 2017). These false positives include “*P. citrulli*-like” but non-pathogenic strains that would lead to incorrect classification of seed lots as infected.

This document assesses whether the continued use of the ZUP qPCR assay in the SE-qPCR protocol remains scientifically justified, considering recent evidence of cross-reactivity and diagnostic limitations.

Genomic Context of qPCR Markers

The ZUP qPCR assay targets IS1002, a repetitive insertion sequence with multiple copies in the *P. citrulli* genome (Woudt *et al.*, 2009a). Its multi-copy nature results in artificially low C_q values and complicates consistent threshold setting across laboratories. In contrast, Contig 21 and Contig 22 are single-copy targets that were identified through an AFLP-based approach using 78 *P. citrulli* strains and 16 outgroup *Paracidovorax* species (Woudt *et al.*, 2009b). Contig 21 is located within gene Aave_0034, which encodes a heavy metal translocating P-type ATPase, while Contig 22 lies in the intergenic region between Aave_3759 (a dihydroxy-acid dehydrogenase) and Aave_3760 (an uncharacterized ORF). Importantly, none of these loci, including IS1002, are known to be directly linked to *P. citrulli* virulence.

Removal of ZUP qPCR Assay

A recent case further illustrates the limitations of the ZUP qPCR assay. A non-*P. citrulli* isolate was recovered in 2024 from squash and clustered phylogenetically near *Acidovorax sacchari*. This isolate tested positive with the ZUP qPCR assay, but not with the more specific Contig 21 qPCR assay. Genomic analysis revealed that the Contig 21 target gene was present but diverged significantly from the *P. citrulli* reference (see Figure 1), preventing amplification. Contig 22 was also present in the isolate, with only three mismatches in the amplicon region (see Figure 2), allowing partial detection. In contrast, the ZUP target region showed a perfect sequence match in the amplicon (see Figure 3), explaining the strong ZUP qPCR signal. These findings emphasize

the risk of using the ZUP qPCR assay in diagnostic workflows where specificity is critical. Recent observations from multiple laboratories suggest that isolates with similar genetic properties and detection profiles are being encountered more frequently, further underscoring the concern.

With the current knowledge on the ZUP qPCR assay and its limited specificity, it appears that combining the ZUP qPCR assay with either the Contig 21 or Contig 22 qPCR assay does not provide additional diagnostic value. Instead, such combinations may increase the risk of false positives without improving overall assay performance. Therefore, assay combinations involving ZUP/Contig 21 or ZUP/Contig 22 are not recommended anymore by ISHI.

Given that the Contig 21 and 22 qPCR assays alone provide equivalent sensitivity and clearly superior specificity, the ZUP qPCR assay does not offer additional diagnostic value and is excluded from the SE-qPCR protocol.

REFERENCES

- ISHI (2021). Protocol for the detection of *Acidovorax citrulli* in cucurbit seed using seed extract and real-time PCR (SE-qPCR). Version 2.0. International Seed Federation (ISF), Nyon, Switzerland. <https://worldseed.org/our-work/seed-health/ishi-methods/>
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- Woudt, B., Beerepoot, A., and Carter, L. (2009a). A new PCR method for detection of *Acidovorax citrulli* on seed. *Phytopathology*, **99**, S143.
- Woudt, B., et al. (2009b). Development of specific primers for the molecular detection of *Acidovorax avenae* subsp. *citrulli*. Proceedings of the EPPO Conference on diagnostics, 2009-05-10/15, York, Great Britain.

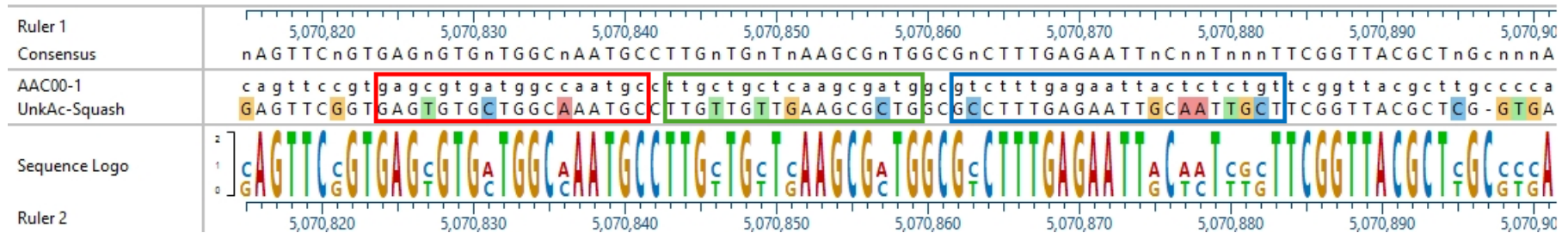


Figure 1. Shown is the amplicon region of **contig21** from the non- *Paracidovorax citrulli* squash isolate (UnkAc-Squash), aligned using Mauve with the reference genome of *P. citrulli* AAC00-1 (GenBank: CP000512). Coloured boxes indicate positions of the diagnostic oligonucleotides: **blue box** – region of Aac F1 primer; **green box** – Aac P1; **red box** – Aac R1 primer.

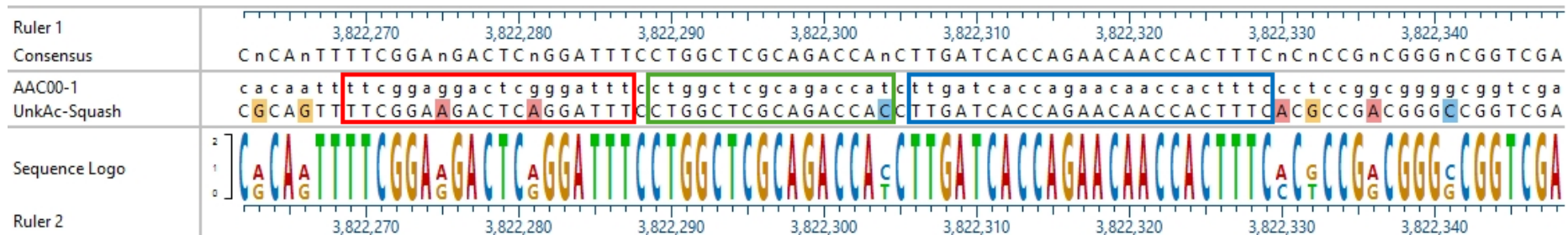


Figure 2. Shown is the amplicon region of **contig22** from the non- *Paracidovorax citrulli* squash isolate (UnkAc-Squash), aligned using Mauve with the reference genome of *P. citrulli* AAC00-1 (GenBank: CP000512). Coloured boxes indicate positions of the diagnostic oligonucleotides: **blue box** – region of Aac F2 primer; **green box** – Aac P2; **red box** – Aac R2 primer.

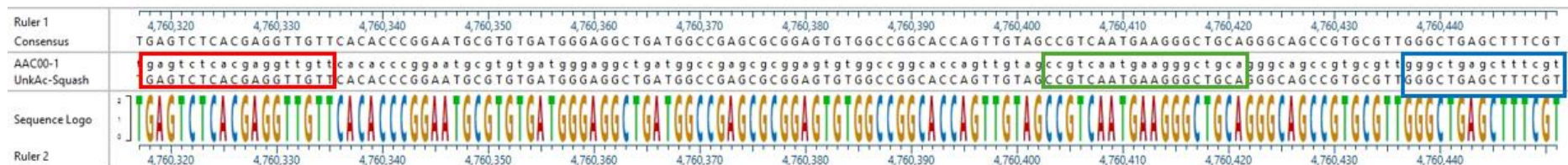


Figure 3. Shown is the amplicon region of **ZUP** from the non- *Paracidovorax citrulli* squash isolate (UnkAc-Squash), aligned using Mauve with the reference genome of *P. citrulli* AAC00-1 (GenBank: CP000512). Coloured boxes indicate positions of the diagnostic oligonucleotides: **blue box** – region of ZUP2551 primer; **green box** – ZUP2551; **red box** – ZUP2549 primer.