High-resolution melting curve analysis: A tool to detect *Ceratocystis eucalypticola* and *C. manginecans* in infected *Eucalyptus*.

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Laboratory Guide:

Complied by Kira Lynn

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Materials:

- Scalpel blade/knife
- CFX96 Real-Time PCR Detection System (BIO-RAD) or any other real-time PCR Detection System that has HRMA capabilities (HRMA software).
- KAPA HRM FAST qPCR Kit (Kapa Biosystems) or kit suited for relevant real-time PCR Detection System.
- Forward primer (CPF.RE) and reverse primer (CPR.RE) at 10 μM
- Distilled deionized water (ddH_2O)
- DNA extraction kits for either infected plant material (DNeasy Plant Mini Kit (Qiagen, Germany)) or fungal culture (Zymo Quick-DNA Fungal/Bacterial Kit (Qiagen, Germany)).
- DNA of selected positive control at 1 ng/μl

Procedure:

Sample preparation:

1. This protocol is better suited for highly infected samples.
    - Samples must be as fresh as possible.
    - For infected wood, choose an area with caramel discolouration. Using a sterile scalpel blade, remove the top layer of infected wood to expose the clean infection below. From here, collect thin shavings for DNA extraction.

Tip: Thin fresh and clean infected wood shavings are optimal for DNA extraction and downstream analysis. Less starting material for DNA extractions is better.

2. Extract DNA from starting material (fungal culture/infected plant) following the manufacturer's protocol and standardize to 1 ng/µl.
3. Prepare reaction mix in a 10 µl volume: 5 µl KAPA HRM FAST Master Mix (or PCR reagent mix of choice), 0.4 µl of each primer, 1 µl 25 mM $MgCl_2$, and 1.2 µl ddH20. Prepare master mix in triplicate for all test samples and a positive and negative control (no-template reaction).
4. Cycling conditions: 3 min at 95 °C, followed by 35 cycles of 5 s at 95 °C, 20 s at 57 °C, and 5 s at 72 °C. Measure fluorescence at the annealing stage using CFX-Manager Software v1.6.
5. A SYBR/FAM fluorescence should be selected for the KAPA HRM FAST Master Mix. Fluorescence detection parameters should be adjusted based on the dye/kit used.
6. Analyse Real-Time PCR results in terms of threshold cycle values (Ct) using default threshold settings. Positive detection of *Ceratocystis* is Ct value ≤ 30 for downstream HRMA, but a weak signal of Ct value ≤ 35 shows detection at low concentrations.
7. Immediately after the real-time PCR amplification, perform a dissociation temperature gradient ranging from 60 °C to 95 °C.
8. Set data acquisition for every 0.2 °C increase in temperature, with a duration of 10s.
9. Utilize the Precision Melt Analysis^{TM} software provided by BioRad for High-Resolution Melt Analysis (HRMA). Or use software relevant to real-time PCR machine used.
10. For HRMA, select "Auto detect melt regions," set "Temperature shift bar height" to the recommended 0.20, maintain "Melt Curve Shape Sensitivity" at the default 50%, and set "Tm Difference Threshold" to the recommended 0.15 degrees.
11. Analyse the data and determine if test samples form a single cluster with the positive control. If so, then samples have been identified.