# Identification of *Alternaria alternata* in combination with analysis of the G143A substitution

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This method was developed by Byron Vega and Megan Dewdney (published in 2014: Distribution of Qol resistance in populations of Tangerine-infecting *Alternaria alternata* in Florida. Plant Disease 98, 67-76.). The PCR-conditions below and the digestion of PCR-products are adapted to suit the reaction enzymes used.

The advantage with this method is that it is species specific for *A. Alternata* and that the PCR product can be cut with a restriction enzyme to reveal the amino acid at position 143 in the gene encoding cytochrome *b*, associated with loss of sensitivity against strobilurins.

***DNA extraction***

- Cut out a leaf disc containing one single lesion (*ca.* 5 mm ø), which include both green and necrotic tissue.
- Washed it twice in sterile distilled water and dried with paper towels.
- Homogenised using five glass beads (3mm) in a 2 ml micro centrifuge tube and shake at 5500 repeats per minute during 30 s (*e.g.* Precellys® 24 Bead Mill Homogenizer, Bertin Technologies).
- Add the extraction buffer. (800 mL of 3% CTAB-EDTA, pH 8 or the amount recommended if using a kit). Shake again as above.
- Follow the CTAB-EDTA protocol or the kit protocol onwards.
- Re-suspend the DNA pellet in 30 µL distilled water if CTAB-protocol or as written in the kit-protocol.
- Dilute the samples to approx. 1 ng DNA $\mu l^{-1}$.

***PCR protocol***

Forward primer: Cytb2f 5′-CTA TGG ATC TTA CAG AGC AC-3′
Reverse primer: DTRcytb2-INTr 5′-GTA TGT AAC CGT CTC CGT C-3′

PCR solution of 20 µl contains 10 µl DNA, 2.75 mM $MgCl_2$ (final concentration), 0.2 mM dNTP, 0.02 µM of each primer, 0.05 U $\mu l^{-1}$ of DreamTaq® DNA Polymerase (Fermentas International Ink, Canada) and 10X DreamTaq™ Green Buffer.

The amplification product is 377 bp, profile I according to Vega and Dewney (2014). They have found a longer product, 1,564 bp, for profile II, but this has not been observed in Sweden so far.

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| Master mix 10µl + 10 µl DNA: | $H_2O$ | 4,5µl |
| | Buffer (10x) | 2µl |
| | dNTP (2 mM) | 2µl |
| | MgCl (25 mM) | 0.6 µl |
| | Primer F (10 nM) | 0.4 µl |
| | Primer R (10 nM) | 0.4 µl |
| | Taq (5 U $\mu l^{-1}$) | 0.1 µl |

PCR condition: 95ºC for 2 min, 40 cycles of 30 s at 95ºC, 30 s at 59ºC and 30 s at 72ºC, followed by a 5 min extension at 72ºC. Separate the products with electrophoresis on 1% agaorose gel stained with suitable dye and visually analyse as under UV-light.

The presence of the G143A substitution is tested using Cleaved Amplified Polymorphic Sequences (CAPS) where the PCR product is digested using the restriction enzyme SatI (Fnu4HI) (Thermo Scientific Inc.). Incubate the reaction volume of 10 µl containing 5 U of enzyme solution and 5 µl of PCR product solution in a 37ºC water bath for two hours. Separate the PCR products in a 1.5% agarose gel.

If the G143A is present in the sample, the length of the digested fragments will be 123 bp and 254 bp for profile I and 254 bp and 1,310 bp for profile II. The fragments of 377 and 1,564 bp remain undigested in wild-type isolates for profile I and profile II respectively.

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